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(71) Applicant *(for all designated States except US)*: MERCK & CO., INC. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: CHEN, Ling [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US). SHIVER, John [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US). BETT, Andrew, J. [CA/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US). CASIMIRO, Danilo, Riguera [PH/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US). CAULFIELD, Michael, J. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US). CHASTAIN, Michael, A. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US). EMINI, Emilio, A. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US).

(74) Common Representative: MERCK & CO., INC.; 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US).

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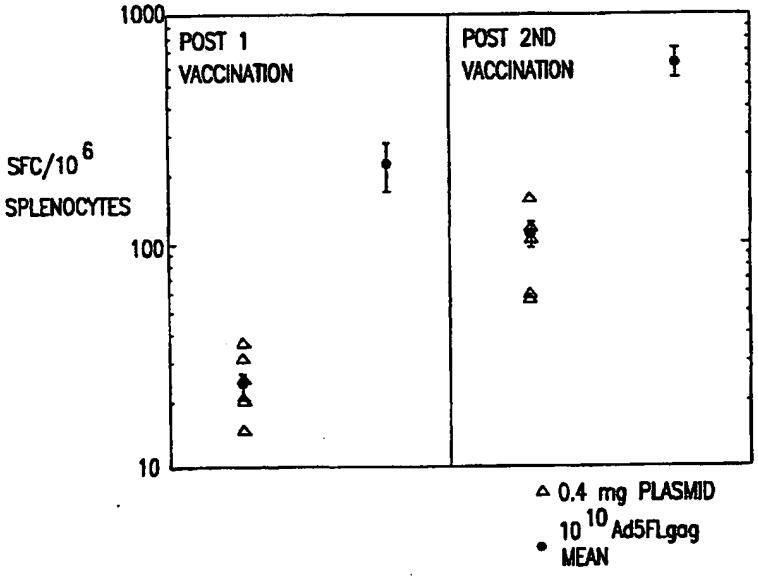
(54) Title: ADENOVIRUS CARRYING GAG GENE HIV VACCINE



(57) Abstract: An adenoviral vector is described which carries a codon-optimized gag gene, along with a heterologous promoter and transcription terminator. This viral vaccine can effectively prevent HIV infection when administered to humans either alone or as part of a prime and boost regime also with a vaccine plasmid.

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ADENOVIRUS CARRYING GAG GENE HIV VACCINE

FIELD OF THE INVENTION

This invention relates to replication deficient adenovirus vectors comprising an optimized human immunodeficiency virus (HIV) gag gene under the control of a strong promoter, which are suitable for vaccines against HIV.

BACKGROUND OF THE INVENTION

Human Immunodeficiency Virus-1 (HIV-1) is the etiological agent of acquired human immune deficiency syndrome (AIDS) and related disorders.

Vaccination is an effective form of disease prevention and has proven successful against several types of viral infection. However, determining ways to present HIV-1 antigens to the human immune system in order to evoke protective humoral and cellular immunity is a difficult task. In AIDS patients, free virus is present in low levels only. Transmission of HIV-1 is enhanced by cell-to-cell interaction via fusion and syncytia formation. Hence, antibodies generated against free virus or viral subunits are generally ineffective in eliminating virus-infected cells.

European Patent Applications 0 638 316 (Published February 15, 1995) and 0 586 076 (Published March 9, 1994), (both assigned to American Home Products Corporation) describe replicating adenovirus vectors carrying an HIV gene, including *env* or *gag*. Various treatment regimens were used with chimpanzees and dogs, some of which included booster adenovirus or protein plus alum treatments.

Infection with HIV-1 is almost always fatal, and at present there are no cures for HIV-1 infection. Effective vaccines for prevention of HIV-1 infection are not yet available. Because of the danger of reversion or infection, live attenuated virus probably cannot be used as a vaccine, and. subunit vaccine approaches have not been successful at preventing HIV infection. Treatments for HIV-1 infection, while prolonging the lives of some infected persons, have serious side effects. There is thus a great need for effective treatments and vaccines to combat this lethal infection.

SUMMARY OF THE INVENTION

This invention relates to a vaccine composition comprising a replication- defective adenoviral vector comprising at least one gene encoding an HIV gag protein, wherein the gene comprises codons optimized for expression in a human, and the gene is operably linked to a heterologous promoter.

Another aspect of this invention relates to an adenoviral vaccine vector comprising: a replication defective adenoviral genome, wherein the adenoviral genome does not have a functional E1 gene, and the adenoviral genome further comprises a gene expression cassette comprising:

i) a nucleic acid encoding a HIV gag protein, wherein the nucleic acid is codon optimized for expression in a human host;

ii) a heterologous promoter is operatively linked to the nucleic acid encoding the gag protein; and

iii) a transcription terminator .

In preferred embodiments, the E1 gene has been deleted from the adenoviral vector, and the HIV expression cassette has replaced the deleted E1 gene. In other preferred embodiments, the replication defective adenovirus genome does not have a functional E3 gene, and preferably the E3 gene has been deleted.

This invention also relates to a shuttle plasmid vector comprising: an adenoviral portion and a plasmid portion, wherein said adenovirus portion comprises: a) a replication defective adenovirus genome which does not have a functional E1 gene; and b) a gene expression cassette comprising: a nucleic acid encoding an HIV gag protein, wherein the nucleic acid is codon optimized for expression in a human host; a heterologous promoter operably linked to the nucleic acid encoding the gag protein; and a transcription terminator.

Other aspects of this invention include a host cell comprising the adenoviral vaccine vectors and/or the shuttle plasmid vectors, methods of producing the vectors comprising introducing the adenoviral vaccine vector into a host cell which expresses adenoviral E1 protein, and harvesting the resultant adenoviral vaccine vectors.

Another aspect of this invention is a method of generating a cellular immune response against an HIV protein in an individual comprising administering to the individual an adenovirus vaccine vector comprising:

a) a replication defective adenoviral vector, wherein the adenoviral vector does not have a functional E1 gene, and

b) a gene expression cassette comprising: i) a nucleic acid encoding an HIV gag protein, wherein the nucleic acid is codon optimized for expression in a human host; ii) a heterologous promoter operatively linked to the nucleic acid encoding the gag protein; and iii) a transcription terminator.

In some embodiments of this invention, the individual is given more than one administration of adenovirus vaccine vector, and it may be given in a regiment accompanied by the administration of a plasmid vaccine. The plasmid vaccine comprises a plasmid encoding a codon-optimized gag protein, a heterologous promoter operably linked to the gag protein nucleic acids, and a transcription terminator. There may be a predetermined minimum amount of time separating the administrations. The individual can be given a first dose of plasmid vaccine, and then a second dose of plasmid vaccine. Alternatively, the individual may be given a first dose of adenovirus vaccine vector, and then a second dose of adenoviral vaccine vector. In other embodiments, the plasmid vaccine is administered first, followed after a time by administration of the adenovirus vector vaccine. Conversely, the adenovirus vaccine vector may be administered first, followed by administration of plasmid vaccine after a time. In these embodiments, an individual may be given multiple doses of the same adenovirus serotype in either viral vector or plasmid form, or the virus may be of differing serotypes.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 is a graph showing the number of gag peptide-specific interferon-gamma secreting splenocytes (x 10^6) from rats which were immunized with gag plasmid or Ad5FLgag.

FIGURE 2 shows serum SEAP (secreted alkaline phosphatase) expression levels in rhesus monkeys following injection with FG Ad5-SEAP or SEAP DNA constructs.

FIGURES 3A, 3B and 3C show anti-HIV gag cytotoxic T lymphocyte responses in three rhesus monkeys vaccinated with FG Ad5 tPAgag. Each panel represents the specific killing response of a particular monkeys (denoted as numbers 92x024 in FIGURE 3A, 94x012 in FIGURE 3B, and 94x025 in FIGURE 3C) at various time points following immunization at 0, 8, and 24 weeks. The abscissa axis shows the effector/target (E/T) ratios of cultured T cells and B cells employed in this assay, while the ordinate axis shows specific lysis values obtained for each sample. Specific lysis values of at least 10% difference between curves ± gag peptide antigen are generally considered significant. The square symbols represent target cells treated with an irrelevant influenza peptide antigen while the circles, triangles, and diamonds represent target cells treated with partial or complete gag peptide pools, respectively.

FIGURES 4A-H show anti-HIV gag cytotoxic T lymphocyte responses in rhesus monkeys vaccinated with FG Ad5FLgag. Figures 4A, B, and C are the first group of monkeys, D,E,and F are the second, and G, H, and I are the third group. Each represents specific killing responses of each monkey receiving the indicated treatment. The abscissa axis shows the effector/target (E/T) ratios of cultured T cells and B cells employed in this assay, while the ordinate axis shows specific lysis values obtained for each sample. Specific lysis values of at least 10% difference between curves ± gag peptide antigen are generally considered significant. The square symbols represent target cells treated with DMSO alone at the same concentration as samples containing peptides while the circles, triangles, and diamonds represent target cells treated with partial (F, G) or complete (H) gag peptide pools, respectively.

FIGURES 5A-H show anti-HIV gag cytotoxic T lymphocyte responses in rhesus monkeys vaccinated with Ad2Flgag priming, followed by either Ad2Flgag or Ad5Flgag boosting. Each panel (Figures 5A-G) represents specific killing responses of a group of three monkeys receiving the indicated treatment. The last panel (Figure 5H) shows responses from two naive monkeys that were not vaccinated. The abscissa axis shows the effector/target (E/T) ratios of cultured T cells and B cells employed in this assay, while the ordinate axis shows specific lysis values obtained for each sample. Specific lysis values of at least 10% difference between curves ± gag peptide antigen are generally considered significant..

FIGURE 6 is the nucleic acid sequence (SEQ.ID.NO.1) of the optimized human HIV-1 gag open reading frame.

FIGURE 7A shows construction of the adenovirus carrying codon-optimized gag. FIGURE 7B shows construction of the adenovirus carrying codon-optimized tPA-gag.

FIGURE 8 is the nucleic acid sequence of the optimized tPA-gag open reading frame.

As used throughout the specification and claims, the following definitions and abbreviations are used:

In general, adenoviral constructs, gene constructs are named by reference to the genes contained therein, such as below:

"tPAgag" refers to a fusion between the leader sequence of the tissue plasminogen activator leader sequence and an optimized HIV gag gene. .

"Ad5-tPAgag" refers to an adenovirus serotype 5 replication deficient virus which carries an expression cassette which comprises a tissue plasminogen

activator leader sequence fused to a codon-optimized gag gene which is under the control of the CMV promoter and contains Intron A.

"Fl" refers to a full length gene.

"Flgag" refers to the full-length optimized gag gene.

"Ad5-Flgag" refers to an adenovirus serotype 5 replication deficient virus which carries an expression cassette which comprises a full length optimized gag gene under the control of the CMV promoter and contains Intron A.

"FG Adenovirus" means a First Generation adenovirus, i.e. a replication deficient adenovirus which has either a non-functional or deleted E1 region, and optionally a non-functional or deleted E3 region.

"Promoter" means a recognition site on a DNA strand to which an RNA polymerase binds. The promoter forms an initiation complex with RNA polymerase to initiate and drive transcriptional activity. The complex can be modified by activating sequences such as enhancers or inhibiting sequences such as silencers.

"Leader" means a DNA sequence at the 5' end of a structural gene which is transcribed along with the gene. This usually results a protein having an N-terminal peptide extension, often referred to as a pro-sequences.

"Intron" as used herein refers to a section of DNA occurring in the middle of a gene which does not code for an amino acid in the gene product. The precursor RNA of the intron is excised and is therefore not transcribed into mRNA not translated into protein.

"Cassette" refers to the a nucleic acid sequence which is to be expressed, along with its transcription and translational control sequences. By changing the cassette, a vector can express a different sequence.

It has been found according to this invention that first generation adenoviral vectors carrying a codon-optimized HIV gag gene regulated with a strong heterologous promoter can be used as human anti-HIV vaccines, and are capable of inducing immune responses.

The adenoviral vector which makes up the backbone of the vaccine construct of this invention is preferably a "first generation" adenoviral vector. This group of adenoviral vectors is known in the art, and these viruses are characterized by being replication-defective. They typically have a deleted or inactivated E1 gene region, and preferably additionally have a deleted or inactivated E3 gene region. In a preferred embodiment of this invention, the first generation replication incompetent adenovirus vector used is a serotype 5 adenovirus containing deletions in E1 (Ad5

base pairs 342-3523) and E3 (Ad5 base pairs 28133 to 30818).. For adenovirus 2 serotype, the E1 deletions are preferably bp 559-3503 and the E3 deletions are preferably 28,812-29,773. (Genbank gb:J01917). Those of skill in the art can easily determine the equivalent sequences for other serotypes, such as serotypes 4, 12, 6, 17, 24, 33, 42, 31, 16.

Adenoviral serotypes 2 and 5, particularly 5 are preferred for use in this invention, since at this point in time, more is known about these serotypes generally than other serotypes, and their complete DNA sequences are known. The prototype serotype 5 adenovirus has been completely sequenced (Chroboczek et al, 1992 *J. Virology* 186:280, which is hereby incorporated by reference.) They also belong to the subgroup C adenoviruses, which are not associated with human or rodent malignancies. However, it is envisioned that any adenovirus serotype can be used in this invention, including non-human ones, as deletion of E1 genes should render all adenoviruses non-tumorogenic. Also it may be advantageous to use a serotype which has less prevalence in the wild, as patients are less likely to have previous exposure (and less pre-existing antibodies) to a rarer serotype.

The adenoviral vectors can be constructed using known techniques, such as those reviewed in Hitt et al, 1997 "Human Adenovirus Vectors for Gene Transfer into Mammalian Cells" *Advances in Pharmacology* 40:137-206, which is hereby incorporated by reference.

In constructing the adenoviral vectors of this invention, it is often convenient to insert them in to a plasmid or shuttle vector. These techniques are known and described in Hitt et al *supra*. This invention specifically includes both the adenovirus and the adenovirus when inserted into a shuttle plasmid.

Viral vectors can be propagated in various E1 complementing cell lines, including the known cell lines 293 and PER.C6. Both these cell lines express the adenoviral E1 gene product. PER.C6 is described in WO 97/00326, published January 3, 1997, which is hereby incorporated by reference. It is a primary human retinoblast cell line transduced with an E1 gene segment that complements the production of replication deficient (FG) adenovirus, but is designed to prevent generation of replication competent adenovirus by homologous recombination. 293 cells are described in Graham et al 1977 *J. Gen. Virol* 36:59-72, which is hereby incorporated by reference.

The HIV gag gene selected to be expressed is of importance to the invention. Sequences for many genes of many HIV strains are publicly available in

GENBANK and primary, field isolates of HIV are available from the National Institute of Allergy and Infectious Diseases (NIAID) which has contracted with Quality Biological (Gaithersburg, MD) to make these strains available. Strains are also available from the World Health Organization (WHO), Geneva Switzerland. In a preferred embodiment of this invention, the gag gene is from an HIV-1 strain (CAM-1; Myers et al, eds. "Human Retroviruses and AIDS: 1995, IIA3-IIA19, which is incorporated by reference). This gene closely resembles the consensus amino acid sequence for the clade B (North American/European) sequence.

Regardless of the HIV gene chosen for expression, the sequence should be "optimized" for expression in a human cellular environment. A "triplet" codon of four possible nucleotide bases can exist in 64 variant forms. That these forms provide the message for only 20 different amino acids (as well as transcription initiation and termination) means that some amino acids can be coded for by more than one codon. Indeed, some amino acids have as many as six "redundant", alternative codons while some others have a single, required codon. For reasons not completely understood, alternative codons are not at all uniformly present in the endogenous DNA of differing types of cells and there appears to exist variable natural hierarchy or "preference" for certain codons in certain types of cells. As one example, the amino acid leucine is specified by any of six DNA codons including CTA, CTC, CTG, CTT, TTA, and TTG (which correspond, respectively, to the mRNA codons, CUA, CUC, CUG, CUU, UUA and UUG). Exhaustive analysis of genome codon frequencies for microorganisms has revealed endogenous DNA of *E. coli* most commonly contains the CTG leucine-specifying codon, while the DNA of yeasts and slime molds most commonly includes a TTA leucine-specifying codon. In view of this hierarchy, it is generally held that the likelihood of obtaining high levels of expression of a leucine-rich polypeptide by an *E. coli* host will depend to some extent on the frequency of codon use. For example, a gene rich in TTA codons will in all probability be poorly expressed in *E. coli*, whereas a CTG rich gene will probably highly express the polypeptide. Similarly, when yeast cells are the projected transformation host cells for expression of a leucine-rich polypeptide, á preferred codon for use in an inserted DNA would be TTA.

The implications of codon preference phenomena on recombinant DNA techniques are manifest, and the phenomenon may serve to explain many prior failures to achieve high expression levels of exogenous genes in successfully transformed host organisms--a less "preferred" codon may be repeatedly present in the

inserted gene and the host cell machinery for expression may not operate as efficiently. This phenomenon suggests that synthetic genes which have been designed to include a projected host cell's preferred codons provide a preferred form of foreign genetic material for practice of recombinant DNA techniques. Thus, one aspect of this invention is an adenovirus vector which specifically includes a gag gene which is codon optimized for expression in a human cellular environment.

The diversity of function that typifies eukaryotic cells depends upon the structural differentiation of their membrane boundaries. To generate and maintain these structures, proteins must be transported from their site of synthesis in the endoplasmic reticulum to predetermined destinations throughout the cell. This requires that the trafficking proteins display sorting signals that are recognized by the molecular machinery responsible for route selection located at the access points to the main trafficking pathways. Sorting decisions for most proteins need to be made only once as they traverse their biosynthetic pathways since their final destination, the cellular location at which they perform their function, becomes their permanent residence.

Maintenance of intracellular integrity depends in part on the selective sorting and accurate transport of proteins to their correct destinations. Over the past few years the dissection of the molecular machinery for targeting and localization of proteins has been studied vigorously. Defined sequence motifs have been identified on proteins which can act as "address labels". Leader or signal peptides such as that from the tissue-specific plasminogen activator protein, tPA, serve to transport a protein into the cellular secretory pathway through the endoplasmic reticulum and golgi apparatus. A number of sorting signals have been found associated with the cytoplasmic domains of membrane proteins such as di-Leucine amino acid motifs or tyrosine-based sequences that can direct proteins to lysosomal compartments. For HIV, transport and extrusion from the cell of viral particles depend upon myristoylation of glycine residue number two at the amino terminus of gag. In some embodiments of the optimized gag gene, the tPA leader sequence has been attached 5' to the structural gene sequence.

The optimized gag gene is incorporated into an expression cassette. The cassette contains a transcriptional promoter recognized by an eukaryotic RNA polymerase, and a transcriptional terminator at the end of the gag gene coding sequence. In a preferred embodiment, the promoter is a "strong" or "efficient" promoter. An example of a strong promoter is the immediate early human

cytomegalovirus promoter (Chapman et al, 1991 *Nucl. Acids Res*19:3979-3986, which is incorporated by reference) with the intron A sequence (CMV-intA), although those skilled in the art will recognize that any of a number of other known promoters, such as the strong immunoglobulin, or other eukaryotic gene promoters may be used, including the EF1 alpha promoter, the murine CMV promoter, Rous sarcoma virus (RSV) promoter, SV40 early/late promoters and the beta-actin promoter . A preferred transcriptional terminator is the bovine growth hormone terminator. The combination of CMVintA-BGH terminator is particularly preferred although other promoter/terminator combinations in the context of FG adenovirus may also be used.

To assist in preparation of the polynucleotides in prokaryotic cells, a shuttle vector version of the adenovirus vector is often prepared. The shuttle vector contains an adenoviral portion and a plasmid portion. The adenoviral portion is essentially the same as the adenovirus vector discussed *supra*,containing adenoviral sequences (with non-functional or deleted E1 and E3 regions) and the gag expression cassette, flanked by convenient restriction sites. The plasmid portion of the shuttle vector often contains an antibiotic resistance marker under transcriptional control of a prokaryotic promoter so that expression of the antibiotic does not occur in eukaryotic cells. Ampicillin resistance genes, neomycin resistance genes and other pharmaceutically acceptable antibiotic resistance markers may be used. To aid in the high level production of the polynucleotide by fermentation in prokaryotic organisms, it is advantageous for the shuttle vector to contain a prokaryotic origin of replication and be of high copy number. A number of commercially available prokaryotic cloning vectors provide these benefits. It is desirable to remove non-essential DNA sequences. It is also desirable that the vectors not be able to replicate in eukaryotic cells. This minimizes the risk of integration of polynucleotide vaccine sequences into the recipients' genome. Tissue-specific promoters or enhancers may be used whenever it is desirable to limit expression of the polynucleotide to a particular tissue type.

In one embodiment of this invention, the shuttle plasmid used is pAD.CMVI-FLHIVgag, was made using homologous recombination techniques. For clinical use, the shuttle vector was rescued into virus in PER.C6 cells. To rescue, the shuttle plasmid was linearized by PacI restriction enzyme digestion and transfected into the PER.C6 cells using the calcium phosphate coprecipitate method. The plasmid in linear form is capable of replication after entering the PER.C6 cells and

virus is produced. The infected cells and media were harvested after viral replication was complete.

Standard techniques of molecular biology for preparing and purifying DNA constructs enable the preparation of the DNA immunogens of this invention.

To ensure a clonal virus population a method of clonal purification was used for clinical material. The virus obtained from transfection of the PER.C6 cells was serially diluted to extinction using 2-fold dilutions. The dilutions were then used to infect PER.C6 cells in 96 well plates using 24 wells for each solution At the end of a 14-day incubation period the wells were scored positive or negative using adenovirus specific PCR and gag ELISA. Virus positive wells at the highest dilutions were selected for expansion. The selected well was the only positive well out of 24 wells plated at that dilution giving 98% assurance of clonality Verification of that endpoint had been reached in the dilution series, and that virus positive wells that had insufficient virus to be detected in the initial screening had not been missed, was obtained by subculturing the original 96 well plated two additional times and re-scoring them This confirmed the clonality of the selected well. The selected virus was designated AD5FLgag.

The adenoviral vaccine composition may contain physiologically acceptable components, such as buffer, normal saline or phosphate buffered saline, sucrose, other salts and polysorbate. One preferred formulation has: 2.5-10 mM TRIS buffer, preferably about 5 mM TRIS buffer; 25-100 mM NaCl, preferably about 75 mM NaCl; 2.5-10% sucrose, preferably about 5% sucrose; 0.01 -2 mM $MgCl_2$; and 0.001%-0.01% polysorbate 80 (plant derived). The pH should range from about 7.0-9.0, preferably about 8.0. One skilled in the art will appreciate that other conventional vaccine excipients may also be used it make the formulation. The preferred formulation contains 5mM TRIS, 75 mM NaCl, 5% sucrose, 1mM $MgCl_2$, 0.005% polysorbate 80 at pH 8.0 This has a pH and divalent cation composition which is near the optimum for Ad5 stability and minimizes the potential for adsorption of virus to a glass surface. It does not cause tissue irritation upon intramuscular injection. It is preferably frozen until use.

The amount of adenoviral particles in the vaccine composition to be introduced into a vaccine recipient will depend on the strength of the transcriptional and translational promoters used and on the immunogenicity of the expressed gene product. In general, an immunologically or prophylactically effective dose of $1x10^7$ to $1x10^{12}$ particles and preferably about $1x10^{10}$ to $1x10^{11}$ particles is administered

directly into muscle tissue. Subcutaneous injection, intradermal introduction, impression through the skin, and other modes of administration such as intraperitoneal, intravenous, or inhalation delivery are also contemplated. It is also contemplated that booster vaccinations are to be provided. Following vaccination with HIV adenoviral vector, boosting with a subsequent HIV adenoviral vector and/or plasmid may be desirable. Parenteral administration, such as intravenous, intramuscular, subcutaneous or other means of administration of interleukin-12 protein, concurrently with or subsequent to parenteral introduction of the vaccine compositions of this invention is also advantageous.

Another aspect of this invention is the administration of the adenoviral vector containing the optimized gag gene in a prime/boost regiment in conjunction with a plasmid DNA encoding gag. To distinguish this plasmid from the adenoviral-containing shuttle plasmids used in the construction of an adenovirus vector, this plasmid will be referred to as a "vaccine plasmid". The preferred vaccine plasmids to use in this administration protocol are disclosed in pending U.S. patent application 09/017,981, filed February 3, 1998 and WO98/34640, published August 13, 1998, both of which are hereby incorporated by reference. Briefly, the preferred vaccine plasmid is designated V1Jns-FL-gag, which expresses the same codon-optimized gag gene as the adenoviral vectors of this invention. The vaccine plasmid backbone, designated V1Jns contains the CMV immediate-early (IE) promoter and intron A, a bovine growth hormone-derived polyadenylation and transcriptional termination sequence as the gene expression regulatory elements, and a minimal pUC backbone (Montgomery et al, 1993 *DNA Cell Biol.* 12:777-783. The pUC sequence permits high levels of plasmid production in *E. coli* and has a neomycin resistance gene in place of an ampicillin resistance gene to provide selected growth in the presence of kanamycin. Those of skill in the art, however, will recognized that alternative vaccine plasmid vectors may be easily substituted for this specific construct, and this invention specifically envisions the use of alternative plasmid DNA vaccine vectors.

The adenoviral vector and/or vaccine plasmids of this invention polynucleotide may be unassociated with any proteins, adjuvants or other agents which impact on the recipients' immune system. In this case, it is desirable for the vector to be in a physiologically acceptable solution, such as, but not limited to, sterile saline or sterile buffered saline. Alternatively, the vector may be associated with an adjuvant known in the art to boost immune responses, such as a protein or other carrier. Agents which assist in the cellular uptake of DNA, such as, but not limited to,

calcium ions, may also be used to advantage. These agents are generally referred to herein as transfection facilitating reagents and pharmaceutically acceptable carriers. Techniques for coating microprojectiles coated with polynucleotide are known in the art and are also useful in connection with this invention.

The adenoviral vaccines of this invention may be administered alone, or may be part of a prime and boost administration regimen. A mixed modality priming and booster inoculation scheme will result in an enhanced immune response, particularly is pre-existing anti-vector immune responses are present. This one aspect of this invention is a method of priming a subject with the plasmid vaccine by administering the plasmid vaccine at least one time, allowing a predetermined length of time to pass, and then boosting by administering the adenoviral vaccine. Multiple primings typically, 1-4, are usually employed, although more may be used. The length of time between priming and boost may typically vary from about four months to a year, but other time frames may be used. In experiments with rhesus monkeys, the animals were primed four rimes with plasmid vaccines, then were boosted 4 months later with the adenoviral vaccine. Their cellular immune response was notably higher than that of animals which had only received adenoviral vaccine. The use of a priming regimen may be particularly preferred in situations where a person has a pre-existing anti-adenovirus immune response.

This invention also includes a prime and boost regimen wherein a first adenoviral vector is administered, then a booster dose is given. The booster dose may be repeated at selected time intervals.

A large body of human and animal data supports the importance of cellular immune responses, especially CTL in controlling (or eliminating) HIV infection,. In humans, very high levels of CTL develop following primary infection and correlate with the control of viremia. Several small groups of individuals have been described who are repeatedly exposed to HIV by remain uninfected; CTL has been noted in several of these cohorts. In the SIV model of HIV infection, CTL similarly develops following primary infection, and it has been demonstrated that addition of anti-CD8 monoclonal antibody abrogated this control of infection and leads to disease progression. This invention uses adenoviral vaccines alone or in combination with plasmid vaccines to induce CTL.

Cellular Immunity Assays for Pre-Clinical and Clinical Research

Another aspect of this invention is an assay which measures the elicitation of HIV-1 protein, including gag-specific cellular immunity, particularly cytotoxic T-lymphocyte (CTL) responses. The "ELIspot" and cytotoxicity assays, discussed herein, measure HIV antigen-specific CD8+ and CD4+ T lymphocyte responses, and can be used for a variety of mammals, such as humans, rhesus monkeys, mice, and rats.

The ELIspot assay provides a quantitative determination of HIV-specific T lymphocyte responses. PMBC cells are cultured in tissue culture microtiter plates. An HIV-1 gag peptide pool that encompasses the entire 500 amino acid open reading frame of gag (50 overlapping 20mer peptides) is added to the cells and one day later the number of cells producing gamma interferon (or another selected interferon) is measured. Gamma interferon was selected as the cytokine visualized in this assay (using species specific anti-gamma interferon monoclonal antibodies) because it is the most common, and one of the most abundant cytokines synthesized and secreted by activated T lymphocytes. For this assay, the number of spot forming cells (SPC) per million PBMCs is determined for samples in the presence and absence (media control) of peptide antigens. This assay may be set up to determine overall T lymphocyte responses (both CD8+ and CD4+) or for specific cell populations by prior depletion of either CD8+ or CD4+ T cells. In addition, ELIspot assays, or variations of it, can be used to determine which peptide epitopes are recognized by particular individuals.

A distinguishing effector function of T lymphocytes is the ability of subsets of this cell population to directly lyse cells exhibiting appropriate MHC-associated antigenic peptides. This cytotoxic activity is most often associated with CD8+ T lymphocytes but may also be exhibited by CD4+ T lymphocytes. We have optimized bulk culture CTL assays in which PBMC samples are infected with recombinant vaccinia viruses expressing antigens (e.g., gag) in vitro for approximately14 days to provide antigen restimulation and expansion of memory T cells that are then tested for cytoxicity against autologous B cell lines treated either with peptide antigen pools. Specific cytotoxicity is measured compared to irrelevant antigen or excipient-treated B cell lines. The phenotype of responding T lymphocytes is determined by appropriate depletion of either CD8+ or CD4+ populations prior to

the cytotoxicity assay. This assay is semi-quantitative and is the preferred means for determining whether CTL responses were elicited by the vaccine.

The following non-limiting Examples are presented to better illustrate the invention.

EXAMPLES

EXAMPLE I

Construction of replication-defective FG-Ad expressing HIV gag antigen

Starting vectors

Shuttle vector pHCMVIBGHpA1 contains Ad5 sequences from bp1 to bp 341 and bp 3534 to bp 5798 with a expression cassette containing human cytomegalovirus (HCMV) promoter plus intron A and bovine growth hormone polyadenylation signal.

The adenoviral backbone vector pAdE1-E3- (also named as pHVad1) contains all Ad5 sequences except those nucleotides encompassing the E1 and E3 region.

Plasmid pV1JNStpaHIVgag contains tPA secretory signal sequence fused to the codon-optimized HIV gag nucleotides under the control of HCMV promoter plus intron A. It is described in pending U.S. patent application 09/017,981, filed February 3, 1998 and WO98/34640, published August 13, 1998, both of which are hereby incorporated by reference.

Plasmid pV1R-FLHIV gag (also named as pV1R-HIVgag-opt) contains codon-optimized full-length HIV gag under the control of the HCMV promoter plus intron A.

Construction of Ad5.tpaHIVgag

1. Construction of adenoviral shuttle plasmid pA1-CMVI-tpaHIVgag containing tPAgag under the control of human CMV promoter and intron A.

The tPAgag insert was excised from pV1JNS-tPAgag by restriction enzymes PstI and XmaI, blunt-ended, and then cloned into EcoRV digested shuttle vector pHCMVIBGHpA1. The orientation of the transgene and the construct were

verified by PCR using the insert specific primers hCMV5'-4 (5'TAG CGG CGG AGC TTC TAC ATC 3' SEQ.ID.NO. __) and Gag3'-1 (5'ACT GGG AGG AGG GGT CGT TGC 3' SEQ.ID.NO.__), restriction enzyme analysis (RcaI, SspBI), and DNA sequencing spanning from CMV promoter to the initiation of the gag.

2. Homologous recombination to generate shuttle plasmid form of recombinant adenoviral vector pAd-CMVI-tpaHIVgag containing tpaHIVgag expression cassette.

Shuttle plasmid pA1-CMVI-tpaHIVgag was digested with restriction enzymes BstZ17 and SgrA1 and then co-transformed into *E. coli* strain BJ5183 with linearized (ClaI digested) adenoviral backbone plasmid pAdE1-E3-. One colony was verified by PCR analysis. The vector was transformed to competent *E. coli* HB101 for large quantity production of the plasmid.

3. Generation of recombinant adenovirus Ad.CMVI-tpaHIVgag in 293 cells.

The shuttle plasmid was linearized by restriction enzyme PacI and transfected to 293 cells using $CaPO_4$ method (InVitrogen kit). Ten days later, 10 plaques were picked and grown in 293 cells in 35-mm plates. PCR analysis of the adenoviral DNA indicated 10 out of 10 virus were positive for gag.

4. Evaluation of large scale recombinant adenovirus Ad.CMVI-tpaHIVgag

Clone No.9 was grown into large quantities through multiple rounds of amplification in 293 cells. One lot yielded of 1.7×10^{12} particles and a second lot yielded 6.7×10^{13} particles. The viral DNA was extracted by proteinase K digestion and confirmed by PCR and restriction enzyme (HindIII) analysis. The expression of tpaHIVgag was also verified by ELISA and Western blot analysis of the 293 or COS cells infected with the recombinant adenovirus. The recombinant adenovirus was used for evaluation in mice and rhesus monkeys.

Construction of Ad5.FHIVgag

1. Construction of adenoviral shuttle plasmid pA1-CMVI-FLHIVgag containing full length HIVgag under the control of human CMV promoter and intron A.

The FLHIVgag insert was excised from pV1R-FLHIVgag by restriction enzyme BglII and then cloned into BglII digested shuttle vector pHCMVIBGHpA1. The orientation and the construct were verified by PCR using the

insert specific primers (hCMV5'-4 and Gag3'-1), restriction enzyme analysis, and DNA sequencing.

2. Homologous recombination to generate plasmid form of recombinant adenoviral vector pAd-CMVI-FLHIVgag containing FLHIVgag expression cassette.

Shuttle plasmid pA1-CMVI-FLHIVgag was digested with restriction enzymes BstZ17 and SgrA1 and then co-transformed into *E. coli* strain BJ5183 with linearized (ClaI digested) adenoviral backbone plasmid pAdE1-E3-. Colonies #6 and #7 were verified by PCR analysis. The vectors were transformed to competent *E. coli* HB101 for large quantity production of the plasmid. The plasmids were verified by HindIII digestion.

3. Generation of recombinant adenovirus Ad.CMVI-FLHIVgag in 293 cells.

The pAd plasmids were linearized by restriction enzyme PacI and transfected to 293 cells using Lipofectamine (BRL). Two weeks later, 6 viruses (#6-1.1, 6-1.2, 6-1.3, 7-1.1, 7-1.2, 7-1.3) were picked and grown in 293 cells in 35-mm plates. PCR analysis using the insert specific primers (hCMV5'-4 and Gag3'-1) of the adenoviral DNA verified the presence of HIV gag.

4. Evaluation of large scale recombinant adenovirus Ad.CMVI-FHIVgag

Virus clone #6-1 was grown into large quantities through multiple rounds of amplification in 293 cells. The viral DNA was extracted by proteinase K digestion and confirmed by PCR, restriction enzyme (HindIII, Bgl II, Bst E II, Xho I) analysis. A partial sequencing confirmed the junction between CMV promoter and the 5' end of HIV gag gene. The expression of FLHIVgag was also verified by ELISA and Western blot analysis of the 293 or COS cells infected with the recombinant adenovirus. The recombinant adenovirus was used for evaluation in mice and rhesus monkeys.

Construction of FG adenovirus FL gag. The full-length (FL) humanized gag gene was ligated into an adenovirus-5 shuttle vector, pHCMVIBGHpA1, containing Ad5 sequences from bp 1 to bp 341 and bp 3534 to bp 5798 with a expression cassette containing human CMV promoter plus intron A and bovine growth hormone polyadenylation signal. The orientation was confirmed by restriction enzyme digestion analysis and DNA sequencing. Homologous recombination in *E. coli* was employed using the shuttle plasmid, pA1-CMVI-

FLHIVgag, and adenoviral backbone plasmid, pAdE1-E3-, to generate a plasmid form of the recombinant adenovirus containing the expression regulatory elements and FL gag gene, pAd.CMVI-FHIVgag. Appropriate plasmid recombinants were confirmed by restriction enzyme digestion.

The pAd plasmid containing the gag expression cassette was linearized by restriction enzyme PacI and transfected to 293 cells (or PER.C6 cells for clinical development candidates) using Lipofectamine (BRL). Two weeks later, 6 viruses were picked and grown in 293 cells in 35-mm plates. PCR analysis using the insert specific primers (hCMV5'-4 and Gag3'-1) of the adenoviral DNA verified the presence of HIV gag. Virus clone #6-1 was grown into large quantities through multiple rounds of amplification in 293 cells. The viral DNA was extracted by proteinase K digestion and confirmed by PCR, restriction enzyme (HindIII, BglII, BstEII, XhoI) analysis. A partial sequencing confirmed the junction between CMV promoter and the 5' end of HIV gag gene. Restriction enzyme analysis demonstrated that the viral genome was stable over the course of these passages.

The expression of HIV gag was verified by ELISA and Western blot analysis of the 293 or COS cells infected with the recombinant adenovirus.

EXAMPLE 2

Immunogenicity/Preclinical Efficacy

The "ELIspot" Assay

The ELIspot assay is a quantitative determination of IV-specific T lymphocyte responses by visualization of gamma interferon secreting cells in tissue culture microtiter plates one day following addition of an HIV-1 gag peptide pool that encompasses the entire 500 amino acid open reading frame of gag (50 overlapping 20mer peptides) to PBMC samples. The number of spot forming cells (SPC) per million of PBMVs is determined for samples in the presence and absence (media control) of peptide antigens. The assay may be set up to determine overall T lymphocyte responses (both CD8+ and CD4+) or for specific cell populations by prior depletion of either CD8+ or CD4+ cells. In addition, the assay can be varied so as to determine which peptide epitopes are recognized by particular individuals.

Cytotoxic T Lymphocyte Assays

In this assay, PBMC samples are infected with recombinant vaccinia viruses expressing gag antigen *in vitro* for approximately 14 days to provide antigen

restimulation and expansion of memory T cells. The cells are then tested for cytotoxicity against autologous B cell lines treated with peptide antigen pools. The phenotype of responding T lymphocytes is determined by appropriate depletion of either CD8+ or CD4+ cells.

A. Immune Responses to FG Adenovirus 5 FLgag Vaccine in Rodents

Adenovirus vectors coding for the gag antigen have consistently produced significantly stronger cellular immune responses than plasmid vectors in rodent species. Table 1 (below) shows ELIspot data from mice vaccinated with Ad5FLgag in comparison with plasmid DNA. Spleens from five mice were pooled and the number of gag peptide-specific interferon-gamma secreting cells was determined.

Table 1: Comparison of plasmid and adenovirus vaccination in mice

	SFC/10^6 splenocytes	
	Post 1st Vaccination	Post 2nd Vaccination
10 µg plasmid	68	324
10^5 Ad5Flgag	18	170
10^8 Ad5Flgag	530	5600

Similar enhancements in the cellular responses to gag were also seen in Fischer rats. FIGURE 1 shows the ELIspot data from individual rats vaccinated with 10^{10} particles of adenovirus Ad5FLgag or with 0.4 mg FL gag plasmid. The mean response after one vaccination was 10-fold higher with adenovirus compared to plasmid. Both vaccines gave a boosted signal after a second vaccination, with the adenovirus vaccine signal 5-fold higher than the plasmid signal.

B. Immune Responses to FG Adenovirus 5 FL gag Vaccine in Rhesus Monkeys

Comparative *in vivo* expression of DNA vs. FGAd5 encoding a reporter gene. Adenovirus and plasmid vectors expressing the secreted alkaline phosphatase (SEAP) as a reporter gene were injected into rhesus monkeys to compare the levels of antigen produced by the two forms of vaccination as shown below.

FIGURE 2 shows that at the highest possible plasmid dose (5 mg), the antigen levels are 1,000-fold lower than the levels achieved using 10^{10} particles of adenovirus, a dose which is ten fold lower than the maximum proposed clinical dose.

FG adenovirus-5 FLgag vaccinations of rhesus monkeys. Three Rhesus monkeys were vaccinated at 0, 8, and 24 weeks with 10^{11} particles of FG adenovirus-5tPAgag, an adenoviral vector containing a form of the gag gene with a leader peptide from the tissue-specific plasminogen activator gene at the amino terminus. Data were collected starting at 20 weeks.

FIGURE 3 shows that all three monkeys had developed strong bulk culture cytotoxicity responses against gag peptide sensitized autologous B cell lines following *in vitro* restimulation using vaccinia-gag for two weeks. These responses were persistent at all time points tested although it is unclear whether the final inoculation at 24 weeks improved the cytotoxicities. In all cases killing is observed with at least one partial peptide pool (i.e. 25 peptides from amino terminus, 25 peptides from the carboxyterminus of gag). In every case killing is observed with the full peptide pool (all 50 peptides spanning the full-length gag). ELIspot assays showed high levels of gamma interferon secreting cells (approximately 200-1000 SFC/million PBMCs) over the course of these experiments, and CD4/8 depletion studies indicated that most responding cells were CD8 T lymphocytes, although most vaccines also had significant CD4 T cell responses.

Immunization of rhesus monkeys with FG adenovirus-5 FLgag. Using a protocol similar to that described above, monkeys were vaccinated with a FG adenovirus-5 construct encoding a full-length gag gene (without the tPA leader peptide). This experiment compared a dose titration of vaccine as well as needle vs. biojector (a needleless injector) delivery at most doses. A third feature of this experiment addresses the concerns raised above about the possible negative effects that prior adenovirus immunity may have on adenoviral vector mediated vaccine responses.

While approximately 150 rhesus sera have been tested for anti-adenovirus-5 neutralizing antibody responses, no significant titers have been detected. Rhesus are a poor host for this viral strain, while about 40-60% of humans have significant neutralizing antibody responses (titers from 10-500). For this experiment, two groups of monkeys (6 and 7) were pre-exposed to FG adenovirus-SEAP vectors

thrice and once, respectively, generating a range of neutralizing antibody responses in these monkeys that encompass the range observed in humans.

FIGURES 4A-I show bulk culture cytotoxicity responses of these vaccines at 8 weeks post a single immunization. All monkeys (groups 1-5, Figures 4A-E) that had not been previously exposed to adenovirus-5 showed significant gag-specific cytotoxic responses at all doses using either biojector or needle while 5/6 vaccines that had been preexposed to adenovirus showed cytotoxic responses (groups 6-7, Figure 4F-G). Control animals have remained consistently negative in these assays (e.g., group 8, Figure 4H).

Anti-gag ELIspot responses were also measured in all monkeys at eight weeks. Table 2, below is a summary of these responses that show that nearly all vaccines developed significant gamma-interferon responses to this vaccine, although prior exposure to adenovirus reduced response levels, and a dose response appears to have been obtained with the highest doses giving the best responses. In addition, in this experiment (as well as an independent experiment) no difference was observed for needle vs. biojector delivery of vaccine. CD4 T cell depletion of these samples showed that the ELIspot responses are largely due to CD8 T cells.

Table 2. Anti-gag ELIspot Responses of Rhesus Monkeys Immunized with FG adenovirus FLgag vaccine.

Group	Rhesus #	Injection	Prior Adeno Exposure	SFC/million PBMCs	
				media	gag pool H
1	96R044	Biojector	none	6	663
"	96R045	"	"	0	665
"	96R046	"	"	5	893
2	96R047	Biojector	none	1	20
"	96R048	"	"	1	104
"	96R049	"	"	0	38
3	96R050	Biojector	none	4	18
"	96R051	"	"	1	14
"	96R052	"	"	10	48
4	96R053	Needle	none	1	410

"	96R054	"	"	0	125
"	96R057	"	"	3	186
5	96R058	Needle	none	1	93
"	96R060	"	"	1	41
"	96R062	"	"	0	6
6	940125	Biojector	3X 10^{10} FG aden-5	15	65
"	940132	"	"	11	39
"	940149	"	"	29	93
7	940145	Biojector	1X 10^{10} FG adeno-5	4	258
"	940147	"	"	15	578
"	940217	"	"	23	55
8	96R063	none	none	0	0
"	96R004	"	"	0	1

These and other data show that higher doses of FG adenovirus vaccines elicited ELIspot responses as high as 800-1000 SFC/million PBMCs (see Table 2). These responses are approximate 5-10 fold higher than those obtained using the highest doses of DNA gag vaccines after four injections over a six month time frame (see Table 3 below) indicating that FG adenovirus vaccines are much more potent than DNA vaccines. Importantly, these data also support the finding that repeated injection of adenoviral vector remain effective although somewhat attenuated in the presence of host immune response to adenovirus.

Combined DNA and FG adenovirus vaccinations in rhesus.

DNA priming may enhance the cellular immune response to gag induced by adenovirus vaccination as shown below. Three rhesus monkeys which had been vaccinated four times with 1 mg of gag plasmid were boosted 4 months following the final DNA shot with 10^{11} particles of FG Ad FLgag. The cellular immune responses (measured by ELIspot and denoted as SFC/million PBMCs) to gag peptides in the monkeys primed with DNA and boosted with adenovirus appear significantly higher than adenovirus vaccination alone. The use of a DNA priming regimen may be particularly advantageous in humans who have preexisting anti-adenovirus immune responses.

Table 3. ELIspot Responses in Rhesus Monkeys After Combined DNA and FG adenovirus gag Vaccinations.

	Monkey #	Priming	Vaccine	Week 20 (2 injections)	Week 28 (3 injections)
DNA prime/	92x004	DNA	Ad5tPAgag	106	781
Ad boost	93x027	DNA	Ad5tPAgag	88	660
	93x023	DNA	Ad5tPAgag	560	609
DNA prime/	93x008	DNA	DNA	344	285
DNA boost	93x012	DNA	DNA	NA	NA
	93x016	DNA	DNA	106	99
Naive/Ad	92x024	None	Ad5tPAgag	373	898
	92x012	None	Ad5tPAgag	276	413
	94x025	None	Ad5tPAgag	531	1275
Control	088R	None	None	5	84
	115Q	None	None	0	8

Boost were performed at week 0, 8, and 24.

Table 4. Anti-gag antibody titer (mMU/ml) in Rhesus Monkeys After DNA and FG adenovirus gag Vaccinations.

	Monkey #	Week 0	Week 8 (1 injection)	Week 20 (2 injections)	Week 28 (3 injections)	Week 40
DNA prime/	92x004	25	7616	10133	12170	15892
Ad boost	93x027	114	36666	20523	95114	31437
	93x023	41	11804	12485	38579	17422
DNA prime/	93x008	158	1689	817	3882	1626
DNA boost	93x012	<10	512	216	722	132
	93x016	20	305	451	2731	735
Naive/Ad	92x024	<10	2454	11460	15711	7449
	92x012	<10	2161	5154	27029	8856
	94x025	14	5852	19159	45990	37586

Boost were performed at week 0, 8, and 24.

C. Determination of HIV-Specific T Lymphocyte Responses in HIV+ Humans

In order to qualify the CTL assays, PBMCs from HIV-1-infected patients, classified as long-term nonprogessors (LTNPs) due to their ability to maintain low levels of systemic viremia and high $CD4^+$ T cell counts over a period of years, were used to measure systemic specific CTL responses. As discussed above, several studies have reported that the presence of HIV-1-specific CTL responses in infected individuals appears to correlate well with maintenance of disease-free infection.

Over the course of numerous independent experiments using PBMCs obtained from approximately 40 LTNPs at five different clinical centers, these HIV-infected individuals generally exhibited strong gag-specific ELIspot and cytotoxicity responses that are predominantly mediated by CD8+ T lymphocytes (CD4+ responses are typically extremely low or undetectable in HIV+ individuals). The overall gag-specific ELIspot responses determined in these experiments are summarized below:

HIV+ ELIspot Response Summary

mean (± SD) SFC/million PBMCs (+ gag peptides) = **980** ± 1584

mean (± SD) SFC/million PBMCs (media control) = **24** ± 21

Similar experiments using PBMCs from 16 HIV seronegative individuals did not show significant gag-specific ELIspot or cytotoxicity responses. These ELIspot responses are summarized below:

HIV Seronegative ELIspot Response Summary

mean (± SD) SFC/million PBMCs (+ gag peptides) = **19** ± 28

mean (± SD) SFC/million PBMCs (media control) = **10** ± 14

The ELIspot assay provides a quantitative determination of HIV-specific T lymphocyte responses by visualization of gamma interferon-secreting cells in tissue culture microtiter plates one day following addition of an HIV-1 gag peptide pool that encompasses the entire 500 amino acid open reading frame of gag (50 overlapping 20mer peptides) to PBMC samples. Gamma interferon was selected as the cytokine visualized in this assay (using species specific anti-gamma interferon monoclonal antibodies) because it is the most common, and one of the most abundant

cytokines synthesized and secreted by activated T lymphocytes. For this assay, the number of spot forming cells (SPC) per million PBMCs is determined for samples in the presence and absence (media control) of peptide antigens. This assay may be set up to determine overall T lymphocyte responses (both CD8+ and CD4+) or for specific cell populations by prior depletion of either CD8+ or CD4+ T cells. In addition, ELIspot assays, or variations of it, can be used to determine which peptide epitopes are recognized by particular individuals.

A distinguishing effector function of T lymphocytes is the ability of subsets of this cell population to directly lyse cells exhibiting appropriate MHC-associated antigenic peptides. This cytotoxic activity is most often associated with CD8+ T lymphocytes but may also be exhibited by CD4+ T lymphocytes. We have optimized bulk culture CTL assays in which PBMC samples are infected with recombinant vaccinia viruses expressing antigens (e.g., gag) in vitro for approximately 14 days to provide antigen restimulation and expansion of memory T cells that are then tested for cytoxicity against autologous B cell lines treated either with peptide antigen pools. Specific cytotoxicity is measured compared to irrelevant antigen or excipient-treated B cell lines. The phenotype of responding T lymphocytes is determined by appropriate depletion of either CD8+ or CD4+ populations prior to the cytotoxicity assay. This assay is the best means for determining whether CTL responses were elicited by the vaccine.

EXAMPLE 3
Clinical Trials

The safety and efficacy of a first generation adenovirus type 5 carrying an optimized gag gene alone and as part of a prime/boost protocol with a gag DNA plasmid are tested.

In the initial trial, subjects receive either 1mg or 5mg HIV gag DNA on a 0, 1, 2 month schedule. Equal number so of Ad5 seropositive and seronegative subjects are involved in the study.

In a second trial, Ad5 seropositive and seronegative individuals receive either 10^7 or 10^9 particles per dose on a 0, 6 month schedule. Some of the individuals who have received a single dose of 10^9 particled of Ad5gag will also receive three injections of HIV gag DNA with 10^{11} particles of Ad5 gag. Also,

individuals who are Ad5 seropositive and seronegative naive individuals will receive 10^{11} particles on a 0, 6 month schedule.

Safety and immunogenicity parameters: Each individual is bled for serum prior to day 0 to determine Ad5 neutralization titers and for PBMCs to establish B-LCL lines for bulk CTL determinations. On day 0 and 4 weeks following each dose of Ad5 gag, PBMCs will be drawn to determine CTL using bulk CTL and ELIspot assays. Immunogenicity will also be measured at later time points to assess persistence of response.

Clinical Study Design Summary

1. Phase I study of HIV gag DNA Priming (plasmid only)

GROUP	Vaccine (n)	Placebo (n)	DNA dose	DNA regimen	Ad5 Sero-status
1	18	3	1 mg	0, 1, 2	+
2	18	3	1 mg	0, 1, 2	-
3	18	3	5 mg	0, 1, 2	+
4	18	3	5 mg	0, 1, 2	=
TOTAL	72	12			

2. Dose ranging study of AD5 gag

Group	Vaccine (n)	Placebo (n)	Ad5 Dose	Ad5 sero status
1	8	2	10^7	+
2	8	2	10^7	-
3	8	2	10^9	+
4	8	2	10^9	-
5	15	2	10^{11}	+
6	15	2	10^{11}	-
TOTAL	62	12		

3. Boosting of gag DNA by Ad5 gag

Group	Vaccine	Placebo	Ad5 dose	Ad5 sero-status	DNA dose	DNA regimen
1*	15	2	10^{11}	+	1 mg	0, 1, 2
2*	15	2	10^{11}	-	1 mg	0, 1, 2
3*	15	2	10^{11}	+	5 mg	0, 1, 2
4*	15	2	10^{11}	-	5 mg	0, 1, 2
	60	8				

* represent the same subjects from Phase 1 study, above.

WHAT IS CLAIMED IS:

1. A vaccine composition comprising a replication defective adenoviral vector comprising at least one gene encoding a HIV gag protein which is codon optimized for expression in a human, and the gene is operably linked to a heterologous promoter and transcription terminator.

2. An adenoviral vaccine vector comprising:

a) a replication defective adenoviral vector, wherein the adenoviral vector does not have a functional E1 gene, and further comprising:

b) a gene expression cassette comprising:

i) a nucleic acid encoding a gag protein which is codon optimized for expression in a human host;

ii) a heterologous promoter operatively linked to the nucleic acid encoding the gag protein; and

iii) a transcription terminator .

3. A vector according to Claim 2, wherein the E1 gene has been deleted from the adenoviral vector.

4. A vector according to Claim 3, wherein the gene expression cassette has replaced the deleted E1 gene.

5. A vector according to Claim 3, wherein the adenovirus vector does not have a functional E3 gene.

6. A vector according to Claim 5 wherein the E3 gene has been deleted from the replication defective adenoviral vector.

7. A vector according to Claim 6 comprising adenoviral 5 sequences deleted of E1 region base pairs (bp) 342-3523 and deleted of E3 region bp 28,133-30,818.

8. A vector according to Claim 6 comprising adenoviral 2 deleted of E1 region bp 559-3503 and E3 region bp 28,812-29,773.

9. A vector according to Claim8 comprising the sequence given in FIGURE 6.

10. A vector according to Claim 8 wherein the sequence is tPA-gag.

11. A vector according to Claim 2 further comprising a physiologically acceptable carrier.

12. An adenoviral vaccine composition for producing an immune response against human immunodeficiency virus (HIV) in a human comprising:

a) adenovirus serotype 5 sequences bp 1 to bp 341 and bp 3534 to 5798; and

b) a gene expression cassette, located 3' to adenovirus sequence bp 341, comprising:

i) a nucleic acid encoding gag which is codon-optimized and optionally has the tPA leader sequence at its 5' end;

ii) a human CMV promoter plus intron A operatively linked to the nucleic acid encoding gag; and

iii) a bovine growth hormone transcription terminator.

13. A plasmid vector comprising:

a) an adenoviral portion comprising an adenoviral vector according to Claim 2; and

b) a plasmid portion.

14. A cell comprising an adenoviral vector of Claim 2.

15. A method of producing the vector of Claim 2 comprising introducing the adenoviral vector of Claim 2 into a host cell which expresses adenoviral E1 protein, and harvesting the resultant adenoviral vectors.

16. A method according to Claim 15 wherein the cell is a 293 cell or PER.C6 cell.

17. A method of generating a cellular immune response against an HIV protein in an individual comprising administering to the individual at least one adenovirus vaccine vector and a vaccine plasmid,

wherein said adenovirus vaccine vector comprises

a) a replication defective adenoviral vector, wherein the adenoviral vector does not have a functional E1 gene, and

b) a gene expression cassette comprising: i) a nucleic acid encoding gag protein optimized for expression in a human host; ii) a heterologous promoter operatively linked to the nucleic acid encoding the gag protein; and iii) a transcription terminator wherein said vaccine plasmid comprises: irus portion comprises

a) a gene expression cassette comprising: a nucleic acid encoding a gag protein, wherein the nucleic acid is codon optimized for expression in a human host;

b) a promoter; and

c) a transcription terminator

wherein the vaccine plasmid does not contain any adenoviral genes.

18. A method according to Claim 17 comprising administering a vaccine plasmid to the individual, and after a predetermined minimum amount of time has passed, administering an adenovirus vaccine vector to the individual.

19. A method according to Claim 17 comprising administering an adenovirus vaccine vector to the individual, and after a predetermined minimum amount of time has passed, administering a vaccine plasmid to the individual.

20. A method according to Claim 14 comprising administering an adenovirus vaccine vector to the individual, and after a predetermined minimum amount of time has passed, re-administering an adenovirus vector to the individual.

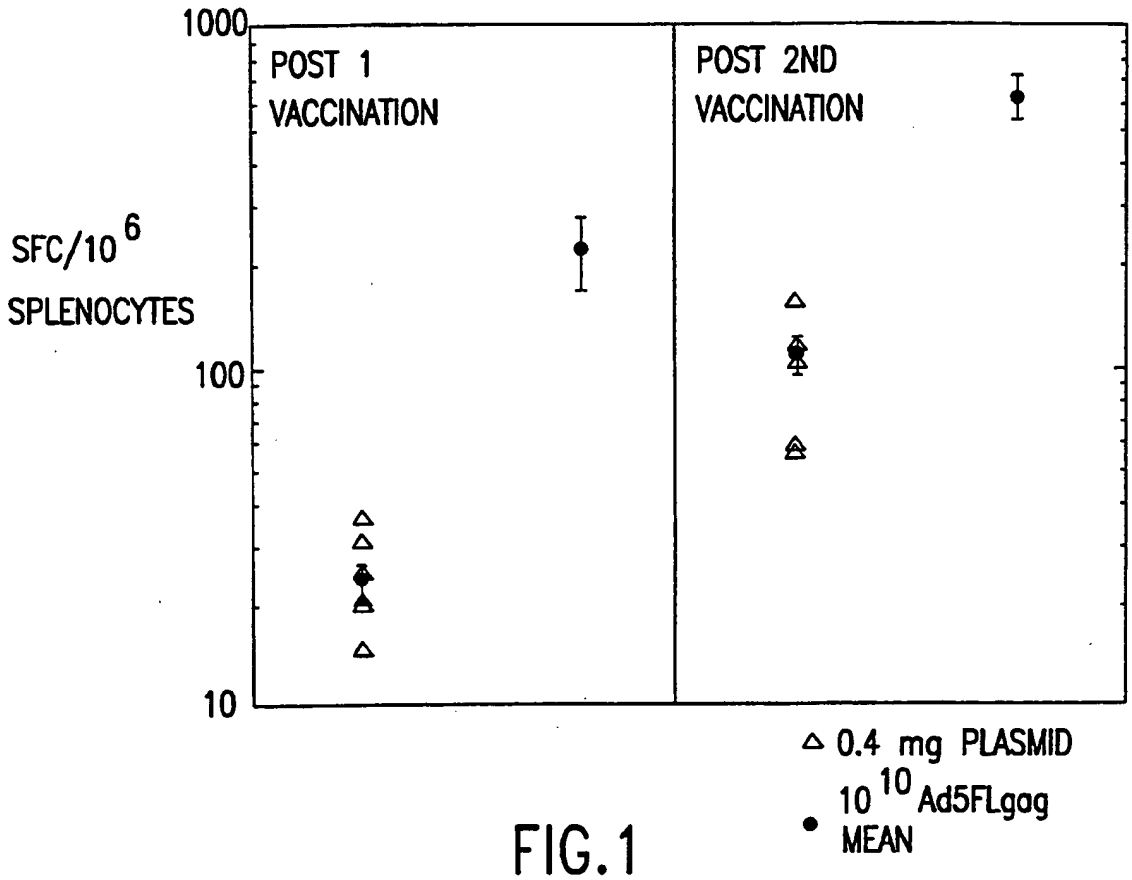

1000
100
10
SFC/10 6
SPLENOCYTES
POST 1
VACCINATION
POST 2ND
VACCINATION
△ 0.4 mg PLASMID
10 10 Ad5FLgag
• MEAN

FIG.1

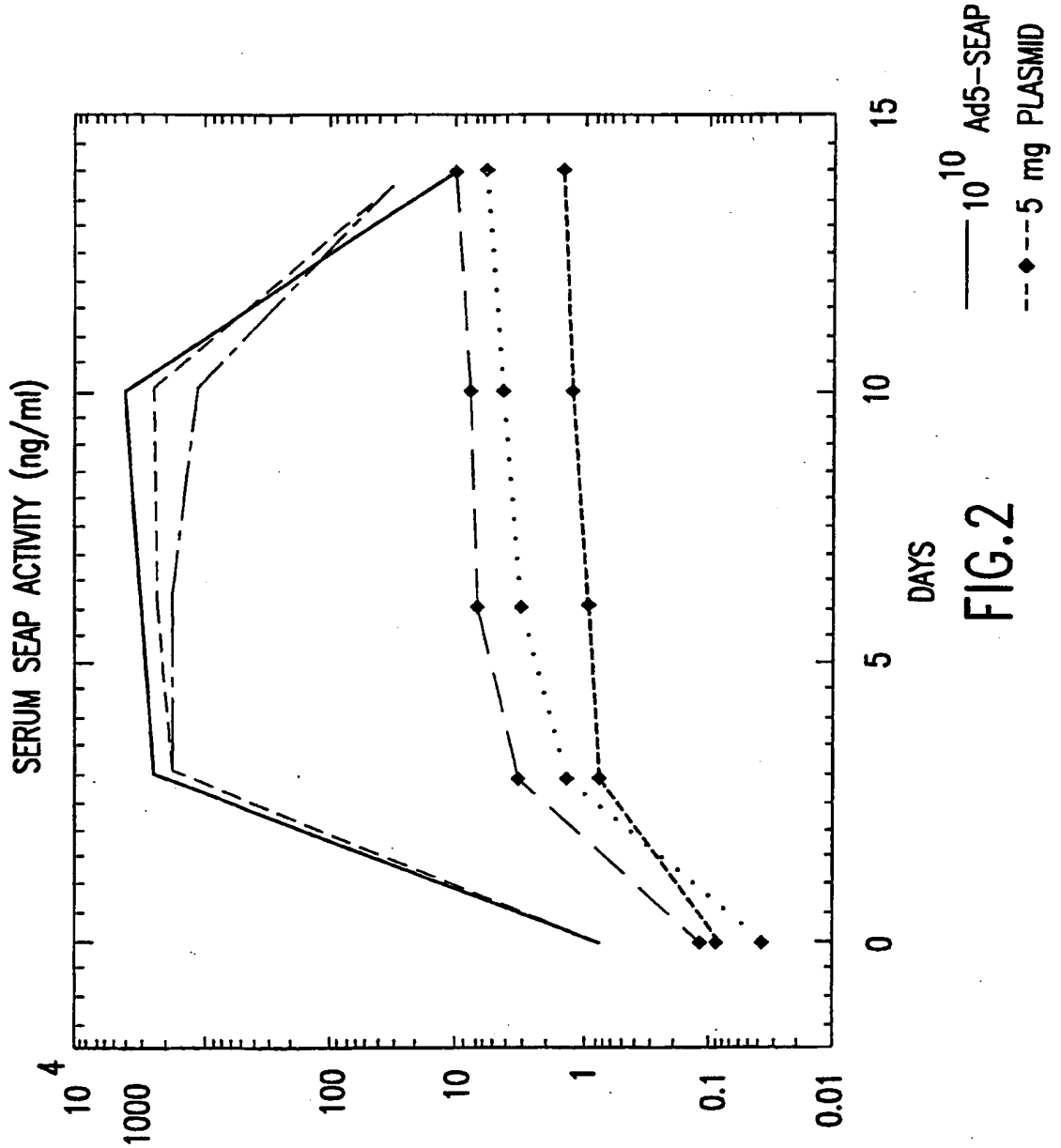

SERUM SEAP ACTIVITY (ng/ml)
10^4
1000
100
10
1
0.1
0.01
0
5
10
15
DAYS
10^{10} Ad5-SEAP
5 mg PLASMID

FIG.2

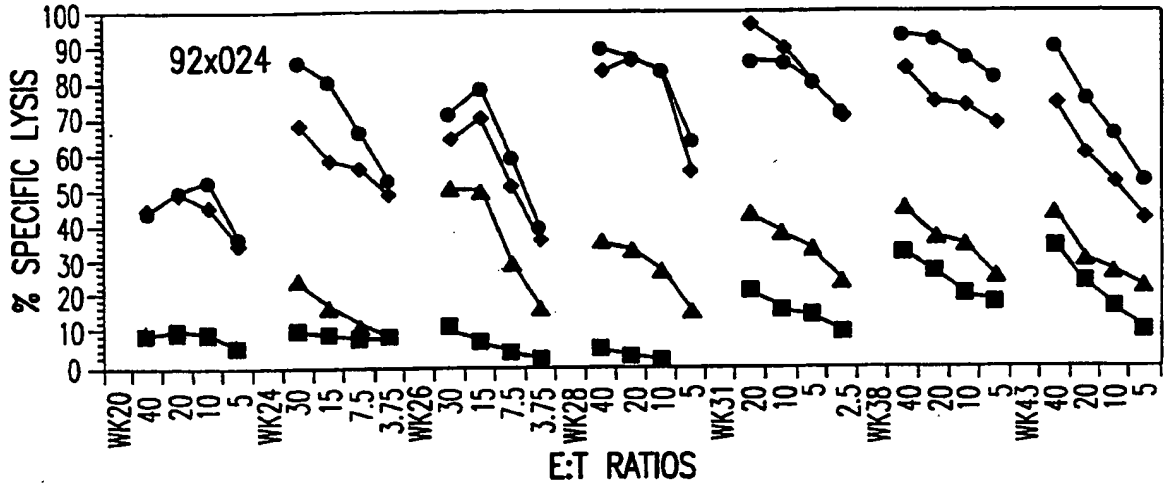


FIG. 3A

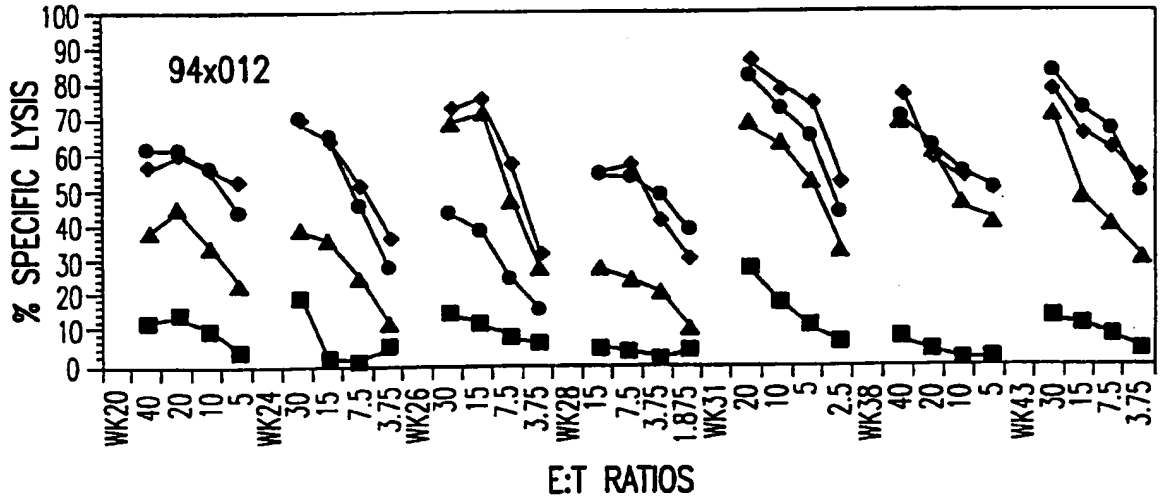


FIG. 3B

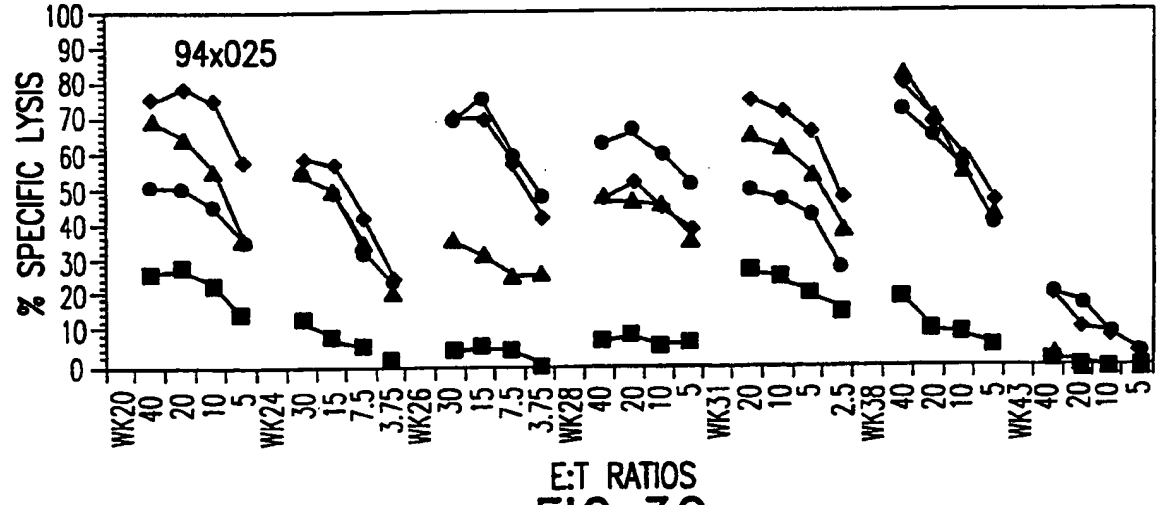


FIG. 3C

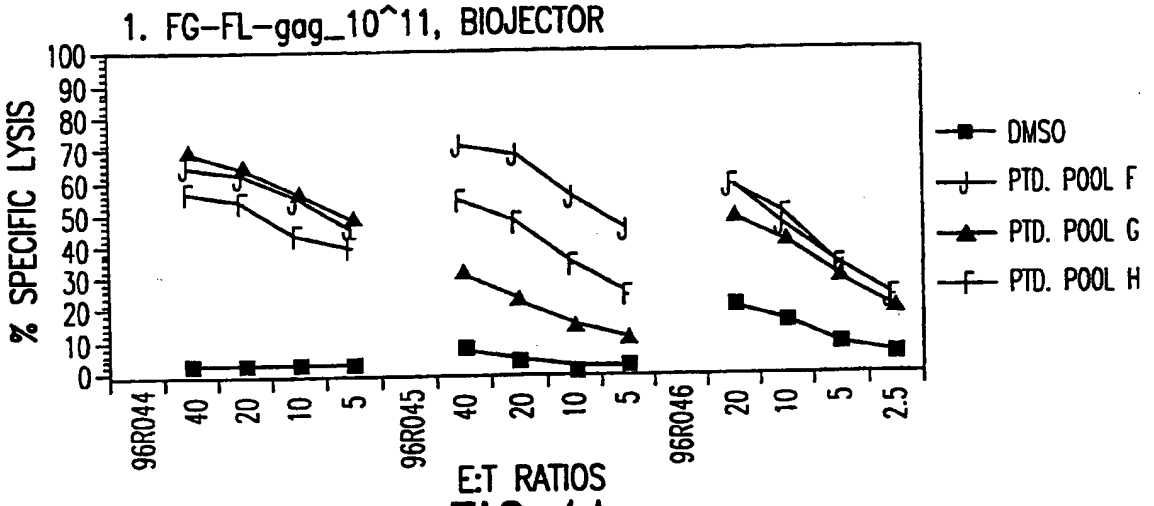


FIG.4A

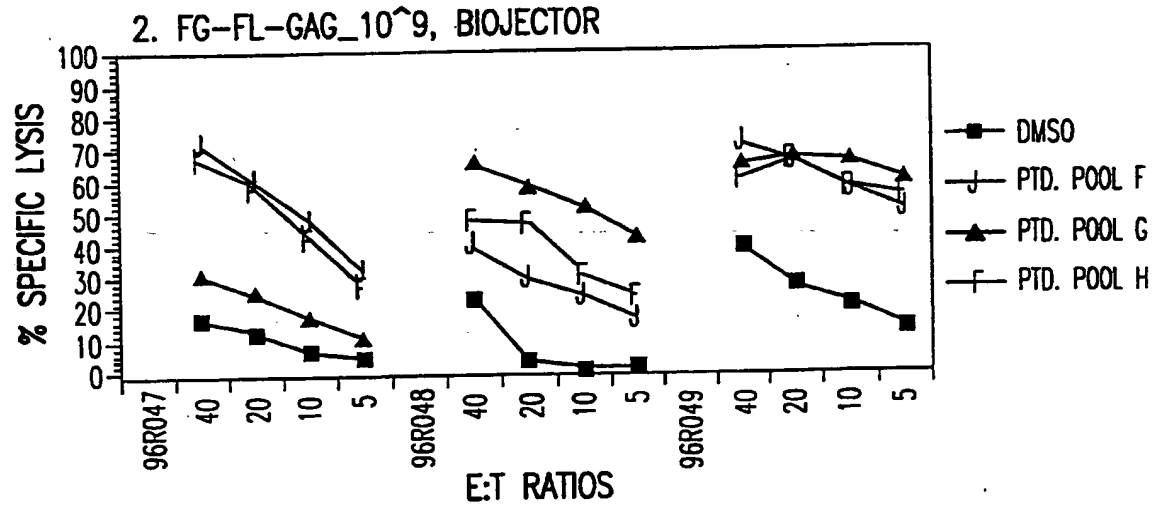


FIG.4B

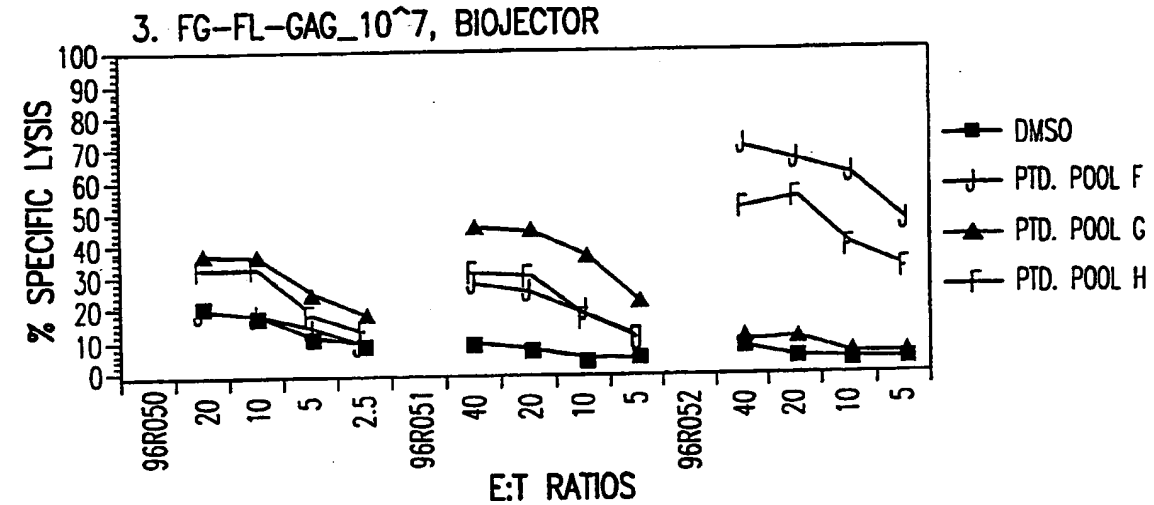


FIG.4C

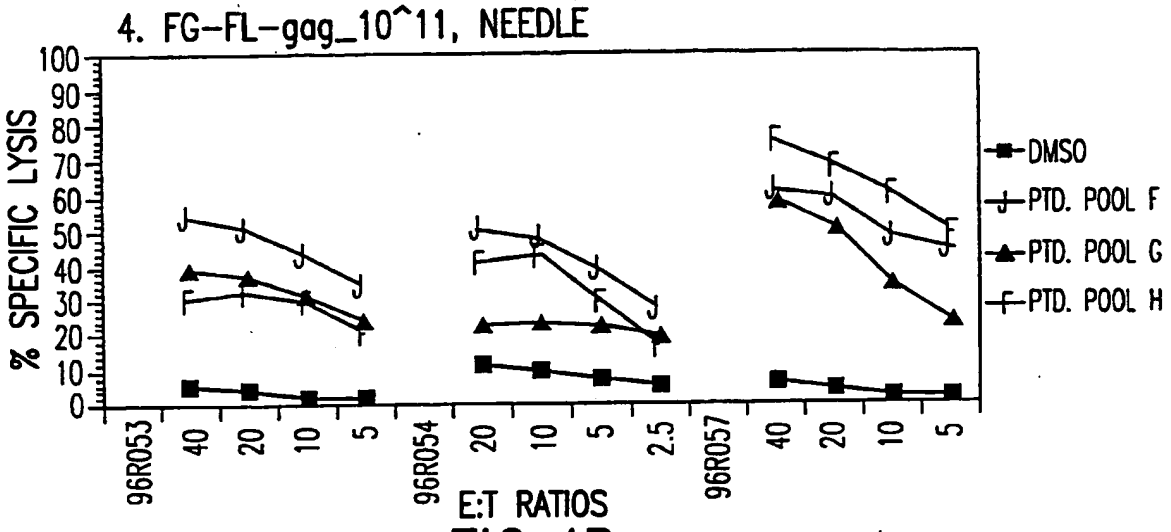


FIG.4D

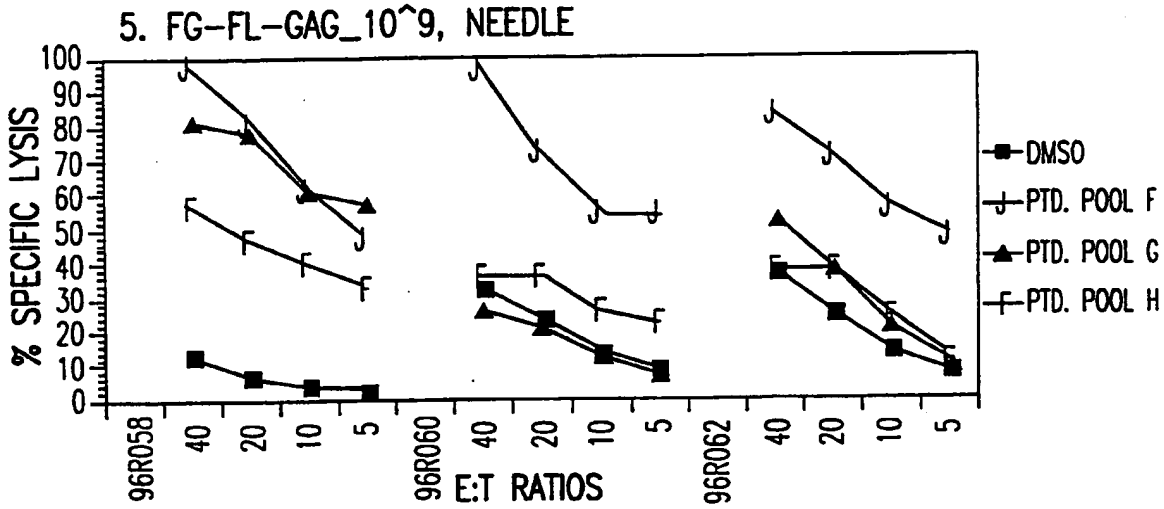


FIG.4E

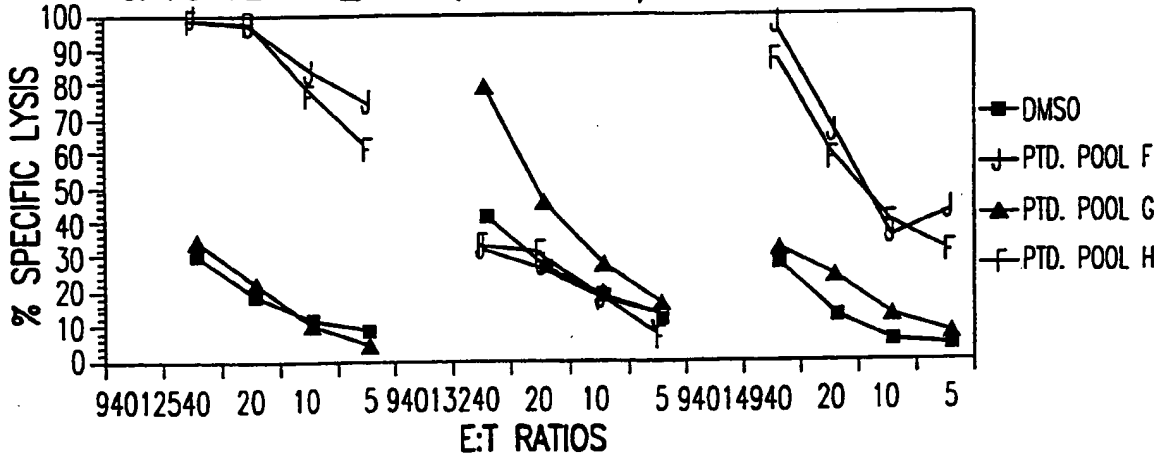
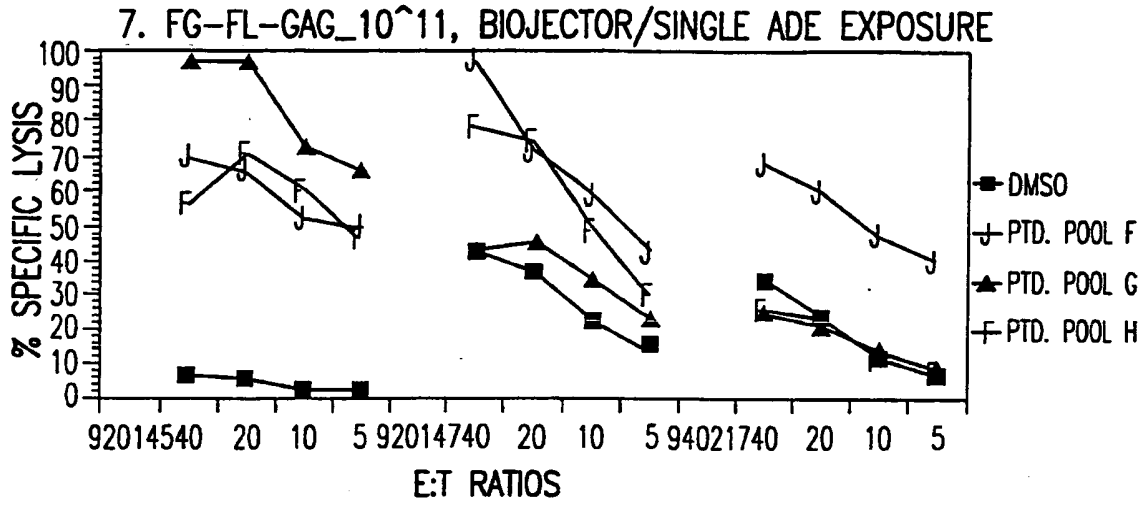


FIG.4F


7. FG-FL-GAG_10^11, BIOJECTOR/SINGLE ADE EXPOSURE
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100
90
80
70
60
50
40
30
20
10
0
92014540 20 10 5 92014740 20 10 5 94021740 20 10 5
E:T RATIOS
DMSO
PTD. POOL F
PTD. POOL G
PTD. POOL H

FIG.4G

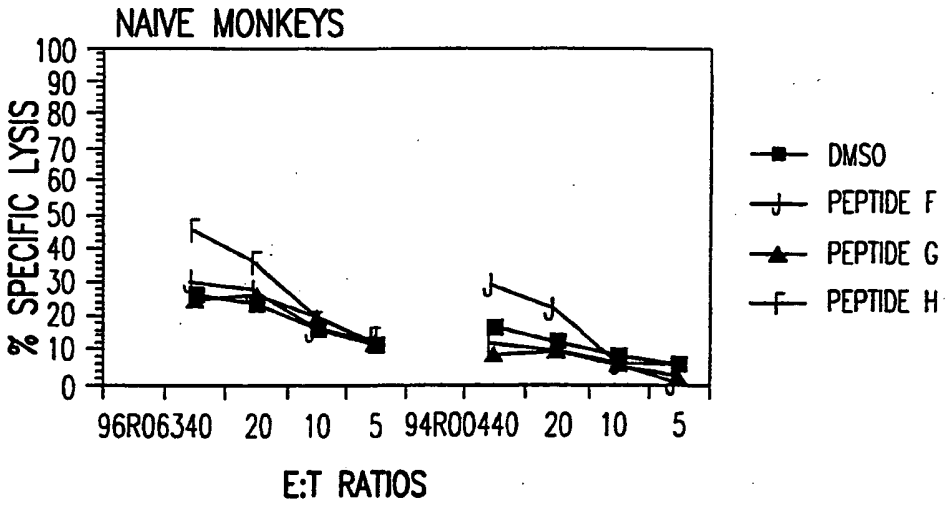

NAIVE MONKEYS
% SPECIFIC LYSIS
100
90
80
70
60
50
40
30
20
10
0
96R06340 20 10 5 94R00440 20 10 5
E:T RATIOS
DMSO
PEPTIDE F
PEPTIDE G
PEPTIDE H

FIG.4H

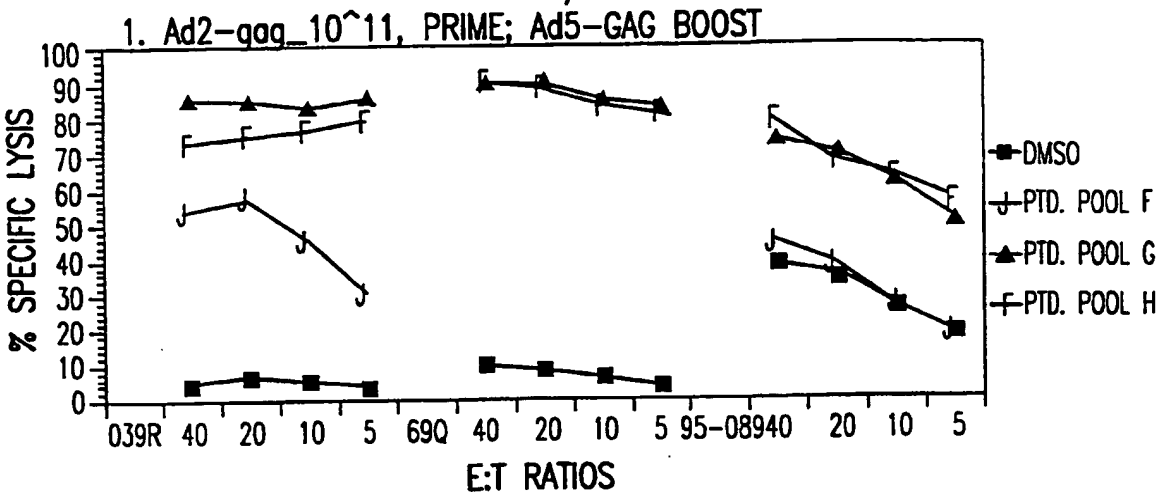


FIG.5A

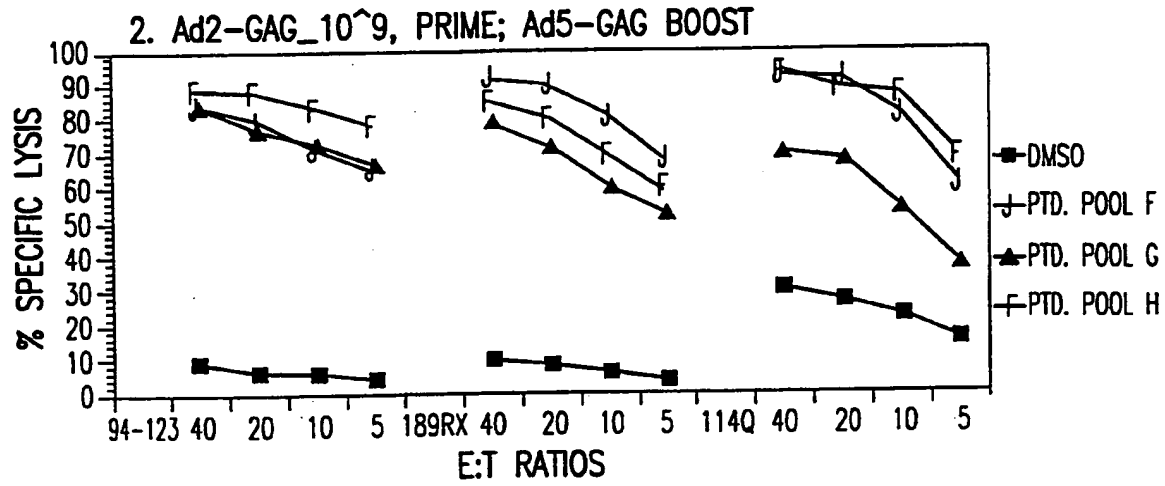


FIG.5B

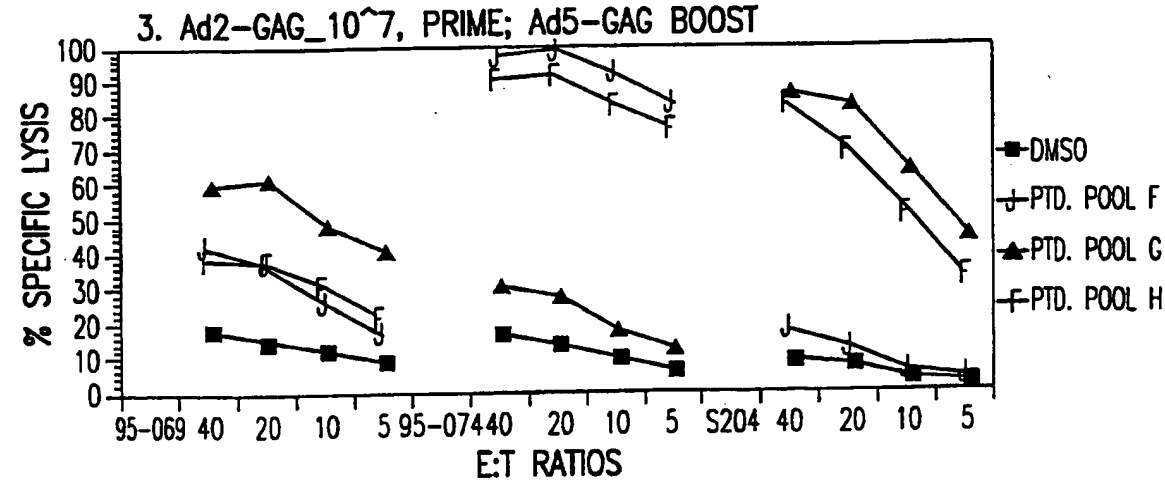


FIG.5C

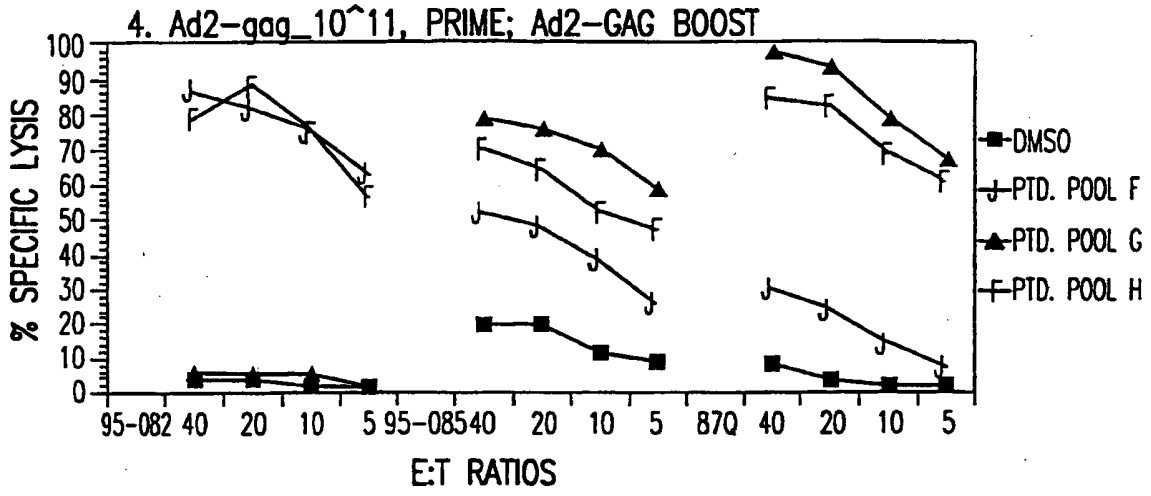


FIG.5D

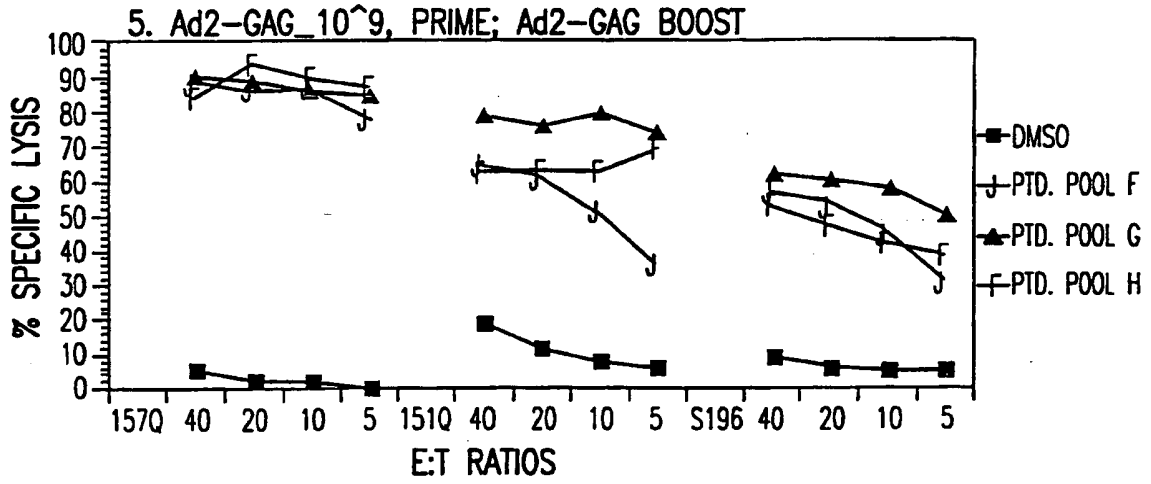


FIG.5E

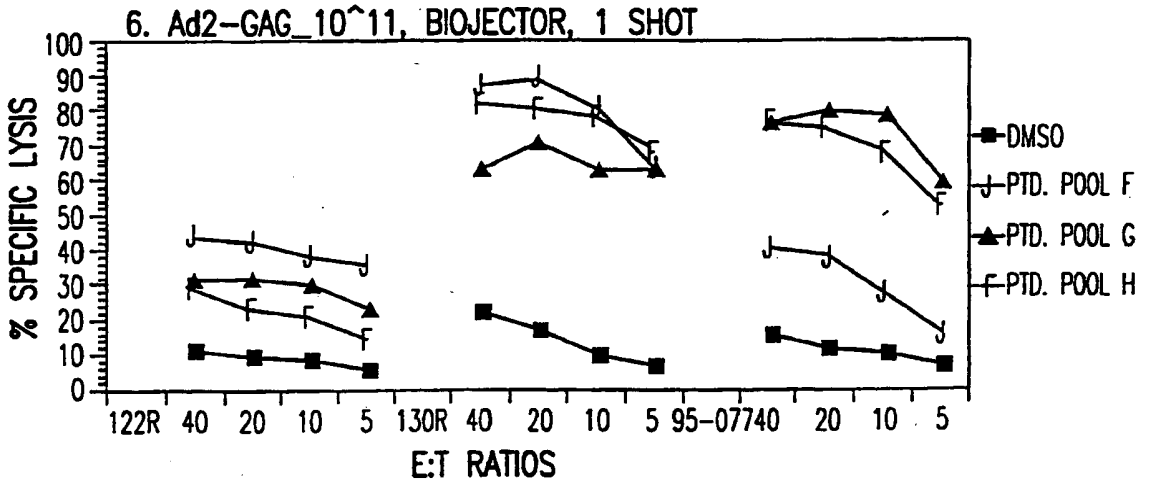


FIG.5F

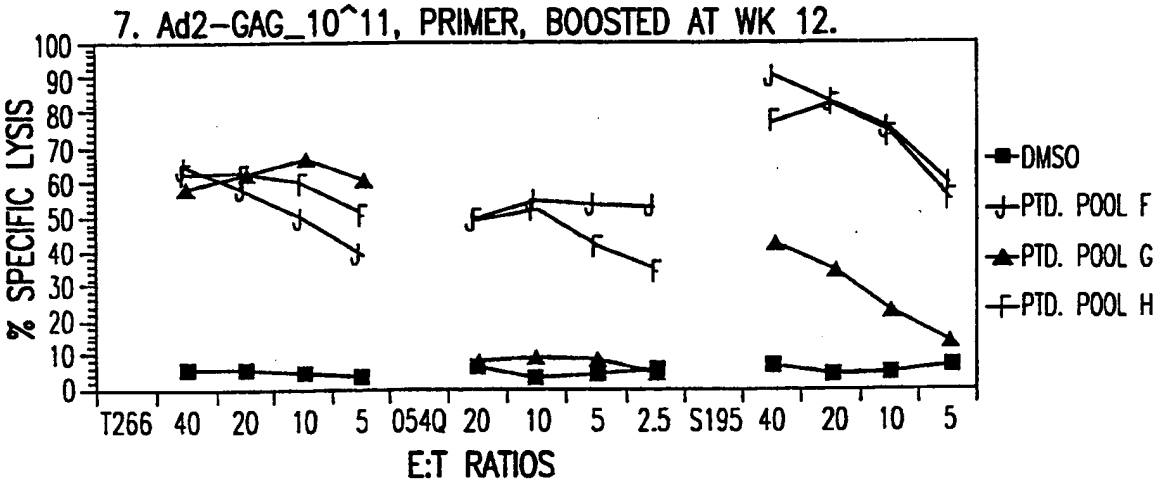

7. Ad2-GAG_10^11, PRIMER, BOOSTED AT WK 12.
% SPECIFIC LYSIS
100
90
80
70
60
50
40
30
20
10
0
T266 40 20 10 5 054Q 20 10 5 2.5 S195 40 20 10 5
E:T RATIOS
DMSO
PTD. POOL F
PTD. POOL G
PTD. POOL H

FIG.5G

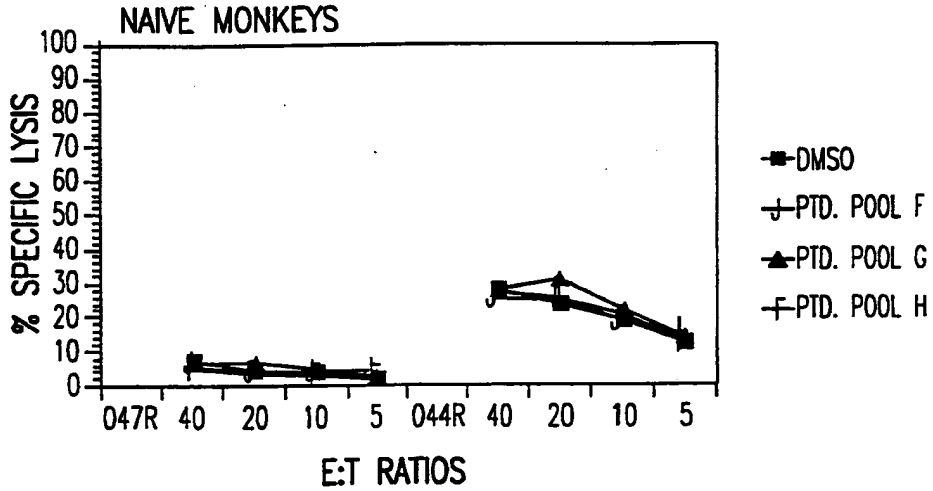

NAIVE MONKEYS
% SPECIFIC LYSIS
100
90
80
70
60
50
40
30
20
10
0
047R 40 20 10 5 044R 40 20 10 5
E:T RATIOS
DMSO
PTD. POOL F
PTD. POOL G
PTD. POOL H

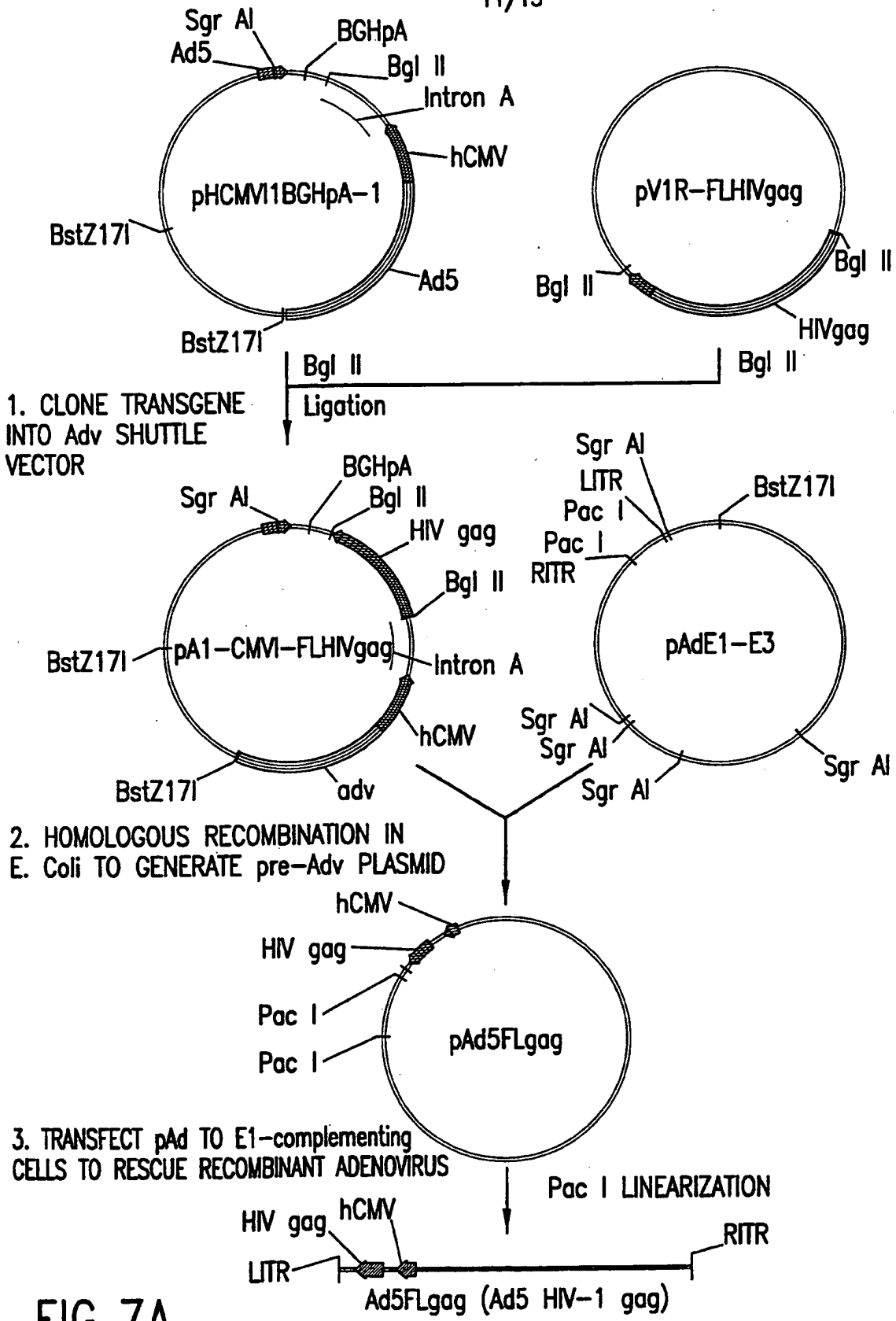
FIG.5H

Optimized HIV-1 (CAM1) *gag* orf

```
   1 AGATCTACCA TGGGTGCTAG GGCTTCTGTG CTGTCTGGTG GTGAGCTGGA
  51 CAAGTGGGAG AAGATCAGGC TGAGGCCTGG TGGCAAGAAG AAGTACAAGC
 101 TAAAGCACAT TGTGTGGGCC TCCAGGGAGC TGGAGAGGTT TGCTGTGAAC
 151 CCTGGCCTGC TGGAGACCTC TGAGGGGTGC AGGCAGATCC TGGGCCAGCT
 201 CCAGCCCTCC CTGCAAACAG GCTCTGAGGA GCTGAGGTCC CTGTACAACA
 251 CAGTGGCTAC CCTGTACTGT GTGCACCAGA AGATTGATGT GAAGGACACC
 301 AAGGAGGCCC TGGAGAAGAT TGAGGAGGAG CAGAACAAGT CCAAGAAGAA
 351 GGCCCAGCAG GCTGCTGCTG GCACAGGCAA CTCCAGCCAG GTGTCCCAGA
 401 ACTACCCCAT TGTGCAGAAC CTCCAGGGCC AGATGGTGCA CCAGGCCATC
 451 TCCCCCCGGA CCCTGAATGC CTGGGTGAAG GTGGTGGAGG AGAAGGCCTT
 501 CTCCCCTGAG GTGATCCCCA TGTTCTCTGC CCTGTCTGAG GGTGCCACCC
 551 CCCAGGACCT GAACACCATG CTGAACACAG TGGGGGGCCA TCAGGCTGCC
 601 ATGCAGATGC TGAAGGAGAC CATCAATGAG GAGGCTGCTG AGTGGGACAG
 651 GCTGCATCCT GTGCACGCTG GCCCCATTGC CCCCGGCCAG ATGAGGGAGC
 701 CCAGGGGCTC TGACATTGCT GGCACCACCT CCACCCTCCA GGAGCAGATT
 751 GGCTGGATGA CCAACAACCC CCCCATCCCT GTGGGGGAAA TCTACAAGAG
 801 GTGGATCATC CTGGGCCTGA ACAAGATTGT GAGGATGTAC TCCCCCACCT
 851 CCATCCTGGA CATCAGGCAG GGCCCCAAGG AGCCCTTCAG GGACTATGTG
 901 GACAGGTTCT ACAAGACCCT GAGGGCTGAG CAGGCCTCCC AGGAGGTGAA
 951 GAACTGGATG ACAGAGACCC TGCTGGTGCA GAATGCCAAC CCTGACTGCA
1001 AGACCATCCT GAAGGCCCTG GGCCCTGCTG CCACCCTGGA GGAGATGATG
1051 ACAGCCTGCC AGGGGGTGGG GGGCCCTGGT CACAAGGCCA GGGTGCTGGC
1101 TGAGGCCATG TCCCAGGTGA CCAACTCCGC CACCATCATG ATGCAGAGGG
1151 GCAACTTCAG GAACCAGAGG AAGACAGTGA AGTGCTTCAA CTGTGGCAAG
1201 GTGGGCCACA TTGCCAAGAA CTGTAGGGCC CCCAGGAAGA AGGGCTGCTG
1251 GAAGTGTGGC AAGGAGGGCC ACCAGATGAA GGACTGCAAT GAGAGGCAGG
1301 CCAACTTCCT GGGCAAAATC TGGCCCTCCC ACAAGGGCAG GCCTGGCAAC
1351 TTCCTCCAGT CCAGGCCTGA GCCCACAGCC CCTCCCGAGG AGTCCTTCAG
1401 GTTTGGGGAG GAGAAGACCA CCCCCAGCCA GAAGCAGGAG CCCATTGACA
1451 AGGAGCTGTA CCCCCTGGCC TCCCTGAGGT CCCTGTTTGG CAACGACCCC
1501 TCCTCCCAGT AAAATAAAGC CCGGGCAGAT CT
```

(SEQ ID NO:1)

FIG.6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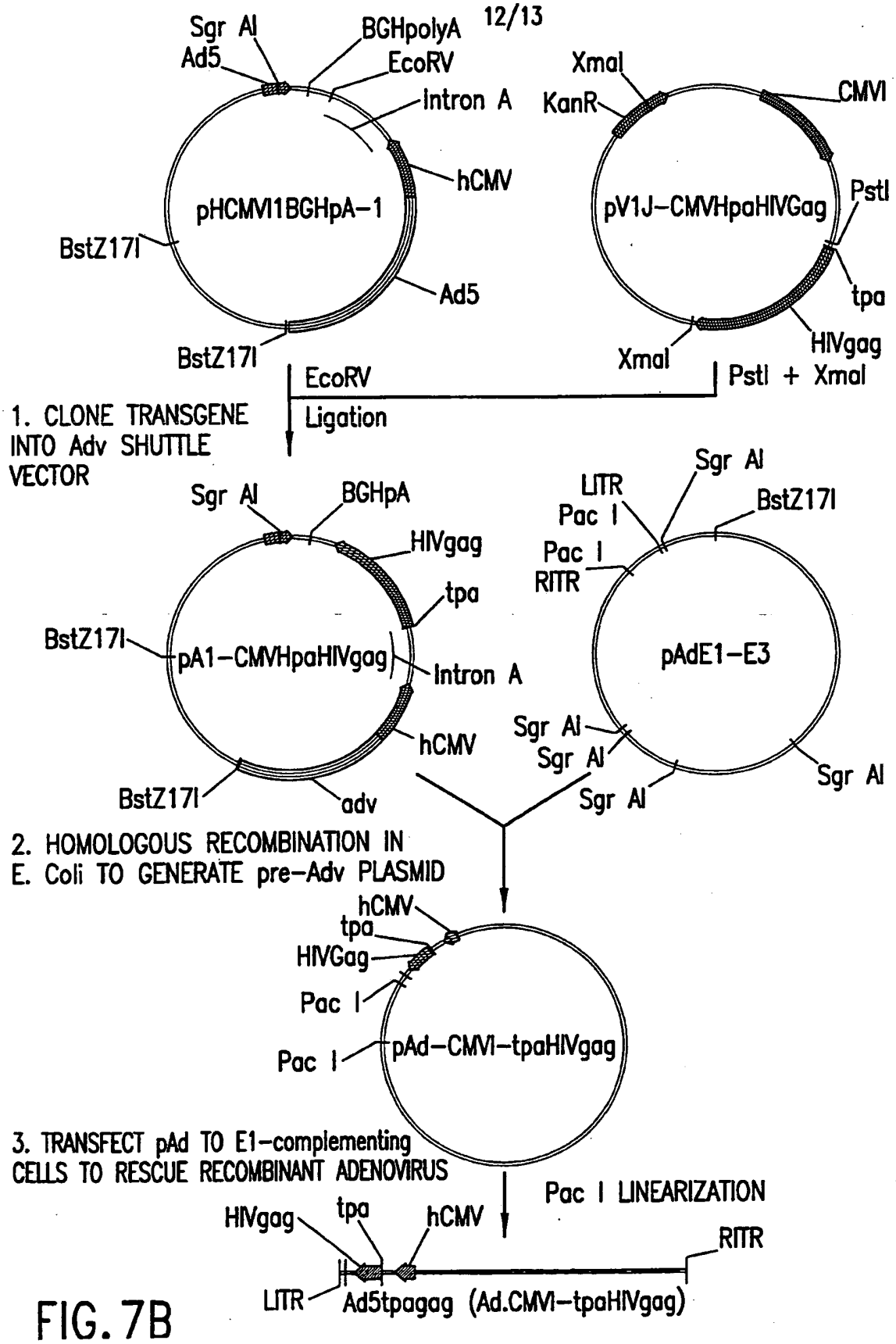
BGHpA
Ad5
Bgl II
Intron A
hCMV
pHCMVI1BGHpA-1
BstZ17I
Ad5
BstZ17I
pV1R-FLHIVgag
Bgl II
Bgl II
HIVgag
Bgl II
Bgl II
Ligation
1. CLONE TRANSGENE INTO Adv SHUTTLE VECTOR
BGHpA
Sgr AI
Bgl II
HIV gag
Bgl II
BstZ17I
pA1-CMVI-FLHIVgag
Intron A
hCMV
BstZ17I
adv
Sgr AI
LITR
Pac I
Pac I
RITR
BstZ17I
pAdE1-E3
Sgr AI
Sgr AI
Sgr AI
Sgr AI
2. HOMOLOGOUS RECOMBINATION IN E. Coli TO GENERATE pre-Adv PLASMID
hCMV
HIV gag
Pac I
Pac I
pAd5FLgag
3. TRANSFECT pAd TO E1-complementing CELLS TO RESCUE RECOMBINANT ADENOVIRUS
Pac I LINEARIZATION
HIV gag
hCMV
RITR
LITR
Ad5FLgag (Ad5 HIV-1 gag)

FIG. 7A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BGHpolyA
Ad5
EcoRV
Intron A
hCMV
pHCMVI1BGHpA-1
BstZ17I
Ad5
BstZ17I
XmaI
KanR
CMVI
pV1J-CMVHpaHIVGag
PstI
tpa
HIVgag
XmaI
EcoRV
PstI + XmaI
Ligation
1. CLONE TRANSGENE INTO Adv SHUTTLE VECTOR
Sgr AI
BGHpA
HIVgag
tpa
BstZ17I
pA1-CMVHpaHIVgag
Intron A
hCMV
BstZ17I
adv
LITR
Pac I
Sgr AI
BstZ17I
Pac I
RITR
pAdE1-E3
Sgr AI
Sgr AI
Sgr AI
Sgr AI
2. HOMOLOGOUS RECOMBINATION IN E. Coli TO GENERATE pre-Adv PLASMID
hCMV
tpa
HIVGag
Pac I
Pac I
pAd-CMVI-tpaHIVgag
3. TRANSFECT pAd TO E1-complementing CELLS TO RESCUE RECOMBINANT ADENOVIRUS
Pac I LINEARIZATION
HIVgag
tpa
hCMV
RITR
LITR
Ad5tpagag (Ad.CMVI-tpaHIVgag)

FIG. 7B

TPA-GAG open reading frame

ATGGATGCAATGAAGAGAGGGCTCTGCTGTGTGCTGCTGCTGTGTGGAGCAGTCTTCG
TTTCGCCCAGCGAGATCTCCATTGTGTGGGCCTCCAGGGAGCTGGAGAGGTTTGCTGT
GAACCCTGGCCTGCTGGAGACCTCTGAGGGGTGCAGGCAGATCCTGGGCCAGCTCCAG
CCCTCCCTGCAAACAGGCTCTGAGGAGCTGAGGTCCCTGTACAACACAGTGGCTACCC
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TTGAGGAGGAGCAGAACAAGTCCAAGAAGAAGGCCCAGCAGGCTGCTGCTGGCACAG
GCAACTCCAGCCAGGTGTCCCAGAACTACCCCATTGTGCAGAACCTCCAGGGCCAGAT
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AAGGCCTTCTCCCCTGAGGTGATCCCCATGTTCTCTGCCCTGTCTGAGGGTGCCACCCC
CCAGGACCTGAACACCATGCTGAACACAGTGGGGGGCCATCAGGCTGCCATGCAGAT
GCTGAAGGAGACCATCAATGAGGAGGCTGCTGAGTGGGACAGGCTGCATCCTGTGCA
CGCTGGCCCCATTGCCCCCGGCCAGATGAGGGAGCCCAGGGGCTCTGACATTGCTGGC
ACCACCTCCACCCTCCAGGAGCAGATTGGCTGGATGACCAACAACCCCCCCATCCCTG
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GTGGACAGGTTCTACAAGACCCTGAGGGCTGAGCAGGCCTCCCAGGAGGTGAAGAAC
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GGGCTGCTGGAAGTGTGGCAAGGAGGGCCACCAGATGAAGGACTGCAATGAGAGGCA
GGCCAACTTCCTGGGCAAAATCTGGCCCTCCCACAAGGGCAGGCCTGGCAACTTCCTC
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FIG.8

SEQUENCE LISTING

<110> Merck & Co., Inc.

<120> ADENOVIRUS CARRYING GAG GENE HIV VACCINE

<130> 20440Y PCT

<150> 60/142,631
<151> 1999-07-06

<150> 60/148,981
<151> 1999-08-13

<160> 4

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<213> Artificial Sequence

<220>
<223> Humanized HIV-1 gag open reading frame

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<211> 1482
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<220>
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cagaacaagt ccaagaagaa ggcccagcag gctgctgctg gcacaggcaa ctccagccag 360
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gtgatcccca tgttctctgc cctgtctgag ggtgccaccc cccaggacct gaacaccatg 540
ctgaacacag tgggggggca tcaggctgcc atgcagatgc tgaaggagac catcaatgag 600
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/18332

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/68; C12P 21/04; C12N 15/00; A61K 39/21; C07H 21/04
US CL : 435/6, 69.7, 320.1; 424/208.1; 536/23.72
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 69.7, 320.1; 424/208.1; 536/23.72

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

USPT, JPAB, EPAB, DWPI; defective adenoviral vector, hiv gag, codon optimized, heterologous promoter, vaccine,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,859,193 A (DEVARE et al) 12 January 1999, column 3, lines 26-37, columns 5-7, Example 1.	1-20
Y	US 5,672,508 A (GYURIS et al) 30 September 1997, column 17, lines 14-56.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 11 SEPTEMBER 2000	Date of mailing of the international search report 18 OCT 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer HANKYEL T. PARK, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)★